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PLANT PHYSIOLOGY

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# THE METABOLISM OF NITROGENOUS COMPOUNDS BY SUNFLOWER CROWN GALL TISSUE CULTURES[1, 2]

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The possibilities for clarifying our understanding of the basic nitrogen metabolism of plant tissues have been improved by the development of plant tissue culture techniques. The influence of various organic and inorganic sources of nitrogen on the growth of sunflower crown gall tissue cultures was studied by Riker and Gutsche (7), who showed that nitrate or urea were excellent sources of nitrogen for the tissue; ammonia also was a fair nitrogen source. Seventeen amino acids, when tested in the presence of an optimal nitrate concentration (0.00836 M), produced progressive inhibition of growth with increasing amino acid concentration; except with arginine, complete inhibition occurred at approximately $10^{-3}$ M. Whereas this inhibition occurred with L-glutamic acid, DL-aspartic acid, and DL-alanine, when these three amino acids were used at concentrations higher than $10^{-3}$ M, good growth was obtained again. Figure 1 shows typical growth curves (7) with two maxima obtained when alanine was employed.

As alanine, glutamic acid, and aspartic acid are actively involved in transamination in a wide variety of plant, animal, and bacterial tissues, the possibility that the interesting growth effects obtained might be concerned with transamination seemed worth exploring.

The experiments described in this paper deal with two aspects of nitrogen metabolism. First were examined the activities of transaminases from sunflower crown gall tissue grown on media containing various levels of alanine, glutamate, or aspartate in the presence of nitrate. Second, tracer experiments were carried out to determine the relative utilization of nitrate or ammonia and also of nitrate and amino acid when both were present in the same medium.

## MATERIALS AND METHODS

TISSUE CULTURE AND MEDIA: The sunflower crown gall tissue used was transferred from cultures isolated and cultivated by Hildebrandt *et al.* (3). Originally the gall was induced by *Agrobacterium tumefaciens* (Smith and Townsend; Bergey *et al.*) on sunflower, *Helianthus annuus* L., var. Giant Russian. These tissues were cultivated on a medium described by Riker and Gutsche (7), which consisted of sucrose as a carbon source, nitrate as a nitrogen source, a carefully balanced salt mixture, and traces of thiamine and pyridoxine. When a semisolid medium was desired, 0.5% agar was added. Variations from this basal medium are specified later. The media were adjusted to pH 6.0 and sterilized by autoclaving.

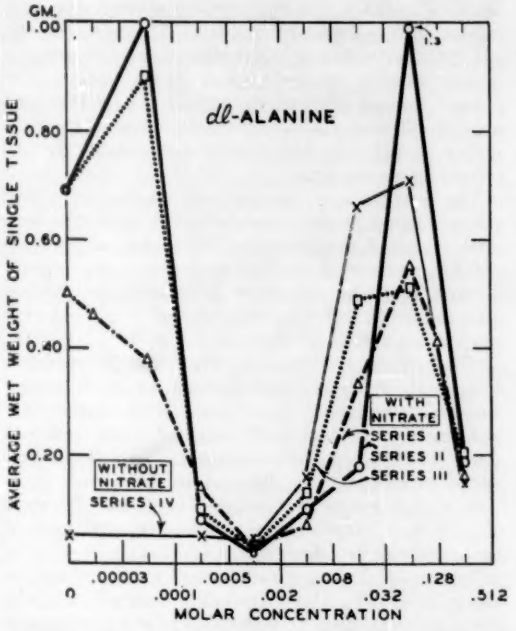


FIG. 1. The weight of growth by sunflower tissue in vitro on basic synthetic media with alanine at concentrations progressively increasing from left to right.

Tissues for transaminase studies were grown in the following manner. Three- to six-week-old tissues grown on the basal agar medium were cut aseptically into pieces about three mm across. Four tissue pieces then were equally spaced on the desired agar medium

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in a six-ounce prescription bottle in which the agar had jelled with the bottle resting on the narrow side. The bottles containing the transferred tissues were placed in an incubation room in the dark at 25–27°C. All operations were performed aseptically.

Tissue used in tracer experiments was grown in liquid media, and stock cultures were transferred aseptically monthly. Transfers of tissue were made by removing one- to two-week-old tissues grown on the basal liquid medium and placing them in 125-ml Erlenmeyer flasks containing 25 ml of the desired medium, or in 2000-ml Florence flasks with larger volumes of media. The flasks containing the transferred tissues were placed on a reciprocating shaker in an incubation room in the dark at 25–27°C.

TRANSAMINASE PREPARATION AND REAGENTS: After a suitable incubation period (about 8 weeks) the tissues were removed from the culture bottles and washed with distilled water to remove adhering agar. Rolling the washed tissue pieces lightly on filter paper dried the tissue and removed any remaining pieces of agar. The tissue was weighed and ground in a glass mortar with quartz sand and 0.05 M borate buffer at pH 8.0; the volume of buffer was adjusted to give the desired rate of transamination. The juice was expressed through cheesecloth, centrifuged for five minutes at $1000 \times g$, the supernatant solution was readjusted to pH 8.0, and this was examined for the transaminase reactions.

The glutamic acid, aspartic acid, alanine, and pyruvic acid used in the transamination determinations were dissolved in glass-redistilled water, adjusted to pH 8.0, and stored at 5°C until used. Alpha-ketoglutaric acid and oxalacetic acid were dissolved in glass-redistilled water immediately before use and were exactly neutralized by the addition of solid $NaHCO_3$.

$N^{15}$-LABELED COMPOUNDS: Inorganic $N^{15}$-enriched compounds purchased from Eastman Kodak Company included potassium nitrate containing 14 atom % $N^{15}$ and ammonium nitrate containing 7.5 atom % $N^{15}$ in the ammonium ion. The ammonium nitrate was converted to ammonium sulfate before use.

DL-alanine and DL-glutamic acid containing 7.5 atom % $N^{15}$ were synthesized by reductive amination of the corresponding keto acids in the presence of a palladium black catalyst under one atmos. of hydrogen at 20°C (9). The amino acids were isolated from the reaction mixture by ion-exchange chromatography on Dowex-50 (10). Glutamic acid was purified as the hydrochloride; alanine was purified as the free amino acid after removing the HCl with an ion-exchange column of amberlite IR-4B. Both amino acids were checked for purity by Kjeldahl nitrogen analysis and by paper chromatography with authentic samples.

CHROMATOGRAPHY: The resolution of tissue hydrolysates into their component amino acids was achieved by ion-exchange chromatography on Dowex-50 (10). An aliquot of each effluent fraction was freed of excess HCl in a heated, evacuated desiccator and was analyzed for amino nitrogen with ninhydrin (6).

Three pairs of amino acids were not entirely resolved by the column of Dowex-50; these were aspartic acid plus serine, glutamic acid plus threonine, and arginine plus phenylalanine. These mixtures were freed of excess HCl by repeated evaporation *in vacuo* followed by drying in a vacuum desiccator containing KOH. The aspartate-serine or the glutamate-threonine mixture in 3 to 5 ml of water was placed on a 10 mm by 120 mm column of IR-4B ion exchange resin (60–180 mesh) in the free base form. Serine or threonine was removed by washing with water and the aspartate or glutamate remaining was eluted with 1 *N* HCl.

The arginine plus phenylalanine sample in 3 to 5 ml of water was placed on a 10 mm by 120 mm column of IRC-50 ion exchange resin (60–180 mesh), which had been equilibrated with a 1 *N* acetate buffer at pH 6.0, and then washed with water to remove excess buffer. The phenylalanine was eluted with water and the arginine with 1 *N* HCl.

All of the fractions containing the same amino acid were pooled, concentrated to dryness *in vacuo*, redissolved in water, reconcentrated to remove excess HCl, dissolved, and stored. Small aliquots of the concentrated fractions were tested for purity by descending chromatography on Whatman No. 1 filter paper beside aliquots of an authentic sample and an authentic sample admixed with the sample to be tested. The chromatograms were developed at 26°C with a solution consisting of 10 gm formic acid, 172 gm water, and 336 gm phenol. At times a second solvent system consisting of 72 gm formic acid, 142 gm water, and 384 gm ethylene glycol diethyl ether was used. The dried papers were sprayed with a solution of 1.5% ninhydrin in water-saturated *n*-butyl alcohol.

NITROGEN ANALYSES: Total nitrogen analyses were made by the semi-micro Kjeldahl method (4, 11). The distilled ammonia was determined either by titration or by the colorimetric Nessler reaction.

For the determination of $N^{15}$ concentration, the ammonia from the Kjeldahl digestion and distillation was converted to $N_2$ with alkaline hypobromite (8). The $N^{15}$ concentration measured in a Consolidated-Nier mass spectrometer, after correction for the normal abundance of $N^{15}$, yielded the atom % $N^{15}$ excess in the sample.

Alpha-amino nitrogen was determined with the ninhydrin titrimetric method of Van Slyke *et al.* (14).

ENZYMATIC PROCEDURES: The following transamination reactions were studied:

(1) L-glutamic acid + oxalacetic acid $\overset{a}{\underset{b}{\rightleftharpoons}}$ alpha-ketoglutaric acid + L-aspartic acid

(2) L-glutamic acid + pyruvic acid $\overset{a}{\underset{b}{\rightleftharpoons}}$ alpha-ketoglutaric acid + L-alanine

Reactions 1a and 1b were studied by methods similar to those of Leonard and Burris (5).

For reaction 1a, 4 ml of the transaminase preparation (pH 8.0) was shaken with 2.0 ml of 0.06 M L-

glutamic acid (pH 8.0) for 10 minutes in a water bath at 32°C. After this equilibration, 2.0 ml of 0.06 M oxalacetic acid were added and the reaction proceeded for 10 minutes. Controls consisted of the transaminase preparation plus glutamic acid and water. The reaction was stopped with one ml of 10% $H_2SO_4$, and the test tubes were placed immediately in a boiling water bath for one hour to destroy residual oxalacetic acid. After cooling, the samples were adjusted to pH 5.0, made to 25 ml volume, and centrifuged. A 2.0-ml aliquot of the supernatant fluid was transferred to a Warburg flask containing 1.0 ml of 0.075 M potassium acid phthalate buffer (pH 5.0) in the main chamber and 10 mg of glutamic acid decarboxylase preparation (13) suspended in 0.5 ml of the same buffer in the side arm. After equilibration for 10 minutes, the decarboxylase was tipped in, and the evolution of $CO_2$ was measured manometrically.

Reaction 1b was measured by placing 1.0 ml of enzyme preparation (pH 8.0) in a Warburg flask containing 1.0 ml of 0.05 M borate buffer (pH 8.0) and 0.5 ml of 0.03 M L-aspartic acid in the main chamber. One side arm contained 0.5 ml of 0.03 M alpha-ketoglutaric acid; the other side arm contained 0.5 ml of aniline citrate (12). After equilibration for 10 minutes at 32°C the alpha-ketoglutarate was tipped in (zero time). The reaction was stopped after 10 minutes, and the oxalacetic acid formed was analyzed by the addition of the aniline citrate from the remaining side arm. The evolved $CO_2$ was measured after 15 minutes. In control flasks 0.5 ml of 2 *N* HCl replaced the aniline citrate, and it was added at zero time to determine the amount of bound $CO_2$. Control flasks with aniline citrate or HCl but no alpha-ketoglutaric acid were used to determine the change in gas pressure arising from endogenous respiration or from volume changes accompanying mixing two liquids. The HCl was added at zero time, the aniline citrate after 10 minutes in these controls.

Reaction 2a, as Reaction 1a, was measured by disappearance of glutamic acid, but pyruvic acid was substituted for oxalacetic acid. For Reaction 2b, 2.0 ml of enzyme sample (pH 8.0) was added to the test tubes containing 1.0 ml of 0.24 M DL-alanine (pH 8.0). After equilibration, 1.0 ml of 0.12 M alpha-ketoglutaric acid was added. After 45 minutes, the samples were boiled, adjusted to pH 5.0, made to a volume of 10.0 ml, and centrifuged. A 2.0-ml aliquot was used for glutamic acid analysis.

TRACER EXPERIMENT PROCEDURE: Certain experiments were performed to investigate the metabolism of $N^{15}$-labeled compounds in tissue cultures. They were relatively long-term experiments followed by analysis of the entire tissue for $N^{15}$ concentration. One to three gm of stock tissue was placed in a 125-ml Erlenmeyer flask containing 25 ml of the desired medium which included an $N^{15}$-labeled compound. After incubation for one week on a reciprocating shaker in a dark incubation room at 25–27°C, the tissue pieces were removed from the flask and rapidly washed five times with distilled water by alternate suspension and decantation. The tissues were weighed, and the wet weights were used to determine percent growth based on the original wet weight at the start of the experiments. The tissue pieces then were killed rapidly by placing them immediately in 100-ml Kjeldahl flasks containing the acid digestion mixture. The samples were digested and distilled, and the amount of ammonia was determined by the Nessler reaction. The ammonia samples then were converted to $N_2$ and analyzed for $N^{15}$.

In other experiments, the tissue was exposed to $N^{15}$-enriched compounds for a relatively short time. Exposure was followed by hydrolysis of the tissue, fractionation of the hydrolysate, and $N^{15}$ analysis of each component. About 250 gm of stock tissue was transferred to a two-liter Florence flask containing 200 or 300 ml of the desired medium. The flask was placed on a reciprocating shaker in a dark incubation room at 25–27°C. After four or eight hours (time is specified for each experiment), the tissues were filtered from the medium and rapidly washed five times by alternate suspension in distilled water and filtration on a Buchner funnel. The tissues were placed immediately in a flask containing 500 ml of 6 *N* HCl, and were hydrolyzed under reflux for 24 hours. The hydrolysate was filtered to remove the humin, and the washed and dried humin was analyzed for total nitrogen and $N^{15}$. The filtrate and washings were concentrated repeatedly *in vacuo* to a syrup to remove HCl. The syrup was taken up in a small amount of water, filtered, and resolved into its component amino acids on a Dowex-50 ion exchange column as previously described. All of the fractions were tested for purity, converted to $N_2$, and analyzed for $N^{15}$.

## EXPERIMENTAL RESULTS

TRANSAMINATION: The data in table I show the effects of DL-alanine, L-glutamic acid, and L-aspartic acid in the culture media on the rates of transamination by the juice from ground tissue cultures. All determinations of transamination were made by measuring glutamic acid or oxalacetic acid manometrically, and rate data are expressed as $Q_T(N)$, i.e., (micromoles of amino acid transaminated)/(mg of nitrogen in the enzyme preparation × hours). The values for "percent transamination" and $Q_T(N)$ represent the average of three or four replicates. The values for "total nitrogen" and "amino nitrogen" represent the average of two or three replicates. To correct for free amino acid absorbed by the tissue but not metabolized, $Q_T(N)$ values were calculated on the basis of total nitrogen minus alpha-amino nitrogen in the enzyme preparation as well as on the basis of total nitrogen.

The concentrations of the amino acids used in the media were those which had been found either to inhibit or to stimulate growth of the tissue cultures. As was previously found by Riker and Gutsche (7), figure 1, the tissue grew well at high and low amino acid concentrations, but poorly at intermediate concentrations. All tissues tested contained an active

TABLE I

RATES OF TRANSAMINATION BY PREPARATIONS FROM SUNFLOWER CROWN GALL TISSUE GROWN ON MEDIA CONTAINING VARIOUS AMINO ACIDS

| MOLAR CONCENTRATION AMINO ACID SUPPLIED IN THE CULTURE MEDIUM | PERCENT TRANSAMINATION | $Q_T(N)$ [a] | $Q_T(N)$ [b] | MG TOTAL N IN ALIQUOT OF ENZYME PREPARATION USED | MG α-AMINO N IN ALIQUOT OF ENZYME PREPARATION USED |
|---|---|---|---|---|---|
| **A. Transamination of glutamate to aspartate** | | | | | |
| None | 6.4 | 142 | 189 | 0.330 | 0.082 |
| 0.00003 DL-alanine | 10.1 | 154 | 195 | 0.413 | 0.087 |
| 0.001 DL-alanine | 9.2 | 187 | 270 | 0.323 | 0.100 |
| 0.045 DL-alanine | 12.0 | 131 | 180 | 0.587 | 0.159 |
| 0.00005 L-glutamate | 7.2 | 157 | 286 | 0.277 | 0.125 |
| 0.01 L-glutamate | 8.9 | 143 | 203 | 0.397 | 0.116 |
| 0.045 L-glutamate | 12.1 | 127 | 185 | 0.634 | 0.200 |
| 0.00005 L-aspartate | 9.9 | 160 | 200 | 0.435 | 0.088 |
| 0.004 L-aspartate | 10.7 | 186 | 268 | 0.397 | 0.122 |
| 0.032 L-aspartate | 15.3 | 138 | 186 | 0.773 | 0.200 |
| **B. Transamination of aspartate to glutamate** | | | | | |
| None | 16.7 | 473 | 608 | 0.032 | 0.007 |
| 0.00003 DL-alanine | 33.6 | 170 | 231 | 0.178 | 0.047 |
| 0.001 DL-alanine | 28.9 | 140 | 226 | 0.185 | 0.070 |
| 0.045 DL-alanine | 26.2 | 141 | 279 | 0.167 | 0.083 |
| 0.00005 L-glutamate | 24.1 | 381 | 690 | 0.057 | 0.026 |
| 0.01 L-glutamate | 13.1 | 278 | 330 | 0.042 | 0.007 |
| 0.045 L-glutamate | 19.3 | 342 | 498 | 0.051 | 0.016 |
| 0.00005 L-aspartate | 8.3 | 244 | 407 | 0.031 | 0.013 |
| 0.004 L-aspartate | 18.5 | 248 | 464 | 0.067 | 0.031 |
| 0.032 L-aspartate | 18.2 | 247 | 358 | 0.066 | 0.021 |
| **C. Transamination of alanine to glutamate** | | | | | |
| None | 9.3 | 43.8 | 53.5 | 0.340 | 0.062 |
| 0.00003 DL-alanine | 9.5 | 46.1 | 54.9 | 0.329 | 0.053 |
| 0.001 DL-alanine | 10.0 | 33.7 | 55.2 | 0.477 | 0.183 |
| 0.045 DL-alanine | 11.2 | 34.0 | 47.9 | 0.526 | 0.153 |
| 0.00005 L-glutamate | 8.2 | 48.0 | 62.4 | 0.273 | 0.063 |
| 0.01 L-glutamate | 10.2 | 39.9 | 60.7 | 0.411 | 0.141 |
| 0.00005 L-aspartate | 5.2 | 29.2 | 37.5 | 0.285 | 0.057 |
| 0.004 L-aspartate | 12.6 | 40.0 | 49.5 | 0.506 | 0.097 |
| 0.032 L-aspartate | 9.3 | 33.8 | 51.8 | 0.440 | 0.153 |

[a] $Q_T(N)$ equals micromoles amino acid transaminated/hours × mg total N of aliquot of enzyme preparation used.
[b] $Q_T(N)$ values calculated on the basis of total N minus α-amino N of aliquot of enzyme preparation used.

glutamic acid–aspartic acid transaminase. The glutamic acid–alanine transaminase was less active; reaction 2a (glutamic acid to alanine) was measurable under conditions described earlier in this paper. By using higher concentrations of reactants and enzyme and extending the reaction time to 45 minutes, reaction 2b could be demonstrated in all cases tested.

The data reveal no direct relationship between transamination capacity of the tissue and growth of the tissue. The observed differences in rates of transamination of tissues grown on media containing different concentrations of glutamic acid, aspartic acid, or alanine were of a lower magnitude and not always in the same direction as changes in growth observed by Riker and Gutsche (7). The data show that as the amino acid concentration in the media was increased, the total tissue nitrogen increased more than the alpha-amino nitrogen. This suggested an improved synthesis of protein by tissues grown on high levels of amino acids.

NITROGEN NUTRITION STUDIED WITH $N^{15}$: If increased protein synthesis by the tissue occurs as indicated by data in table I, is the source of this new protein nitrogen nitrate, amino acid, or both when both are present in the same medium? Cultures containing nitrate and amino acids at different concentrations were grown to determine the relative utilization of mixed nitrogen sources. Ammonia and nitrate mixtures also were studied, as ammonia could be an intermediate in the reduction of nitrate. Each medium was tested with four replicate cultures; each culture flask contained one to three gm of tissue. After growth for one week, the contents of each flask were analyzed separately. The data obtained from these experiments are summarized in table II; parts A and B under each compound represent the results of separate series of experiments.

In the experiments with ammonia, the control medium (Nos. A1 and B1) was the basal medium with nitrate at $8.36 \times 10^{-3}$ M concentration; the nitrate was the sole source of nitrogen and contained 14 atom % $N^{15}$. In media numbers A2, A3, B2, and B3, am-

TABLE II

UTILIZATION OF AMMONIA AND AMINO ACIDS IN THE PRESENCE OF NITRATE BY SUNFLOWER CROWN GALL TISSUE CULTURES *

| NITROGEN SOURCE IN ADDITION TO NITRATE † | PERCENT GROWTH (WET WT.) | PERCENT N & STD. DEV. OF MEAN | ATOM % $N^{15}$ EXCESS & STD. DEV. OF MEAN |
|---|---|---|---|
| Ammonia [a] | | | |
| A. 1. None | 42.2 | 0.103 ± 0.010 | 7.21 ± 0.38 |
| 2. 0.00025 M $NH_3$ [a] (no $NO_3$) | 18.4 | 0.053 ± 0.006 | 0.08 ± 0.051 |
| 3. 0.004 M $NH_3$ [a] (no $NO_3$) | −1.7 | 0.058 ± 0.006 | 2.67 ± 0.12 |
| 4. 0.00025 M $NH_3$ [a, b] | 15.0 | 0.058 ± 0.004 | 0.06 ± 0.007 |
| 5. 0.004 M $NH_3$ [a, b] | 17.7 | 0.099 ± 0.005 | 2.74 ± 0.02 |
| B. 1. None | 23.5 | 0.054 ± 0.004 | 2.86 ± 0.45 |
| 2. 0.00025 M $NH_3$ [a] (no $NO_3$) | 19.2 | 0.045 ± 0.004 | 0.34 ± 0.033 |
| 3. 0.004 M $NH_3$ [a] (no $NO_3$) | 7.1 | 0.086 ± 0.014 | 2.06 ± 0.15 |
| 4. 0.00025 M $NH_3$ [a, b] | 24.0 | 0.070 ± 0.007 | 0.24 ± 0.055 |
| 5. 0.004 M $NH_3$ [a, b] | 12.4 | 0.090 ± 0.006 | 2.18 ± 0.02 |
| DL-alanine | | | |
| C. 1. None (Control) [c] | 59.1 | 0.095 ± 0.003 | 5.80 ± 0.03 |
| 2. 0.1 M DL-alanine | 29.6 | 0.240 ± 0.008 | 0.66 ± 0.033 |
| 3. None (Control) [c] | 42.2 | 0.103 ± 0.010 | 7.21 ± 0.38 |
| 4. 0.00005 M DL-alanine | 27.5 | 0.092 ± 0.017 | 7.10 ± 0.91 |
| D. 1. None (Control) | 23.5 | 0.054 ± 0.004 | 2.86 ± 0.45 |
| 2. 0.1 M DL-alanine | −1.1 | 0.149 ± 0.011 | 0.28 ± 0.096 |
| 3. 0.001 M DL-alanine | 11.8 | 0.066 ± 0.002 | 1.67 ± 0.16 |
| 4. 0.00005 M DL-alanine | 17.1 | 0.046 ± 0.003 | 2.27 ± 0.40 |
| L-glutamic acid | | | |
| E. 1. None (Control) | 42.2 | 0.103 ± 0.010 | 7.21 ± 0.38 |
| 2. 0.1 M L-glutamate | −2.1 | 0.149 ± 0.006 | 0.73 ± 0.041 |
| 3. 0.00005 M L-glutamate | 35.4 | 0.083 ± 0.012 | 6.63 ± 0.07 |
| F. 1. None (Control) | 23.5 | 0.054 ± 0.004 | 2.86 ± 0.45 |
| 2. 0.1 M L-glutamate | 0.2 | 0.108 ± 0.008 | 0.25 ± 0.092 |
| 3. 0.001 M L-glutamate | 14.4 | 0.054 ± 0.002 | 1.92 ± 0.04 |
| 4. 0.00005 M L-glutamate | 13.3 | 0.055 ± 0.006 | 2.27 ± 0.14 |
| L-aspartic acid | | | |
| G. 1. None (Control) | 42.2 | 0.103 ± 0.010 | 7.21 ± 0.38 |
| 2. 0.1 M L-aspartate | −0.8 | 0.181 ± 0.014 | 0.73 ± 0.040 |
| 3. 0.00005 M L-aspartate | 46.8 | 0.082 ± 0.009 | 6.88 ± 0.53 |
| H. 1. None (Control) | 23.5 | 0.054 ± 0.004 | 2.86 ± 0.45 |
| 2. 0.1 M L-aspartate | 5.3 | 0.150 ± 0.014 | 0.30 ± 0.052 |
| 3. 0.001 M L-aspartate | 15.0 | 0.063 ± 0.001 | 1.71 ± 0.01 |
| 4. 0.00005 M L-aspartate | 28.7 | 0.048 ± 0.003 | 2.84 ± 0.02 |
| I. Glycine | | | |
| 1. None (Control) | 23.5 | 0.054 ± 0.004 | 2.86 ± 0.45 |
| 2. 0.1 M glycine | −1.5 | 0.111 ± 0.006 | 0.49 ± 0.108 |
| 3. 0.001 M glycine | 24.8 | 0.067 ± 0.006 | 0.12 ± 0.046 |
| 4. 0.00005 M glycine | 20.0 | 0.050 ± 0.001 | 2.36 ± 0.40 |

* Cultures grown for one week.

† 0.00836 M nitrate (14 atom % $N^{15}$) unless otherwise specified.

[a] Ammonia containing 7.5 atom % $N^{15}$.

[b] 0.00836 M nitrate containing normal N.

[c] No. 1 is control for No. 2; No. 3 is control for No. 4.

monia (7.5 atom % $N^{15}$) at $2.5 \times 10^{-4}$ M and $4.0 \times 10^{-3}$ M concentrations was the sole source of nitrogen. Media numbers A4, A5, B4, and B5 contained as nitrogen sources both unenriched nitrate ($8.36 \times 10^{-3}$ M) and $N^{15}$-enriched ammonia ($2.5 \times 10^{-4}$ M and $4.0 \times 10^{-3}$ M, 7.5 atom % $N^{15}$). In series A the control tissues (No. 1) grew well and contained 7.21 atom % $N^{15}$ excess after growth for one week. Tissues which were given $N^{15}$-labeled ammonia as the sole source of nitrogen (Nos. 2 and 3) showed incorporation of the $N^{15}$ comparable to that of the controls only at the higher ammonia concentration (the $N^{15}$ level of the ammonia supplied in these experiments was only about half that of the nitrate). When unlabeled nitrate was present in addition to the $N^{15}$-labeled ammonia (Nos. 4 and 5), virtually the same $N^{15}$ concentrations were noted as when ammonia alone was the nitrogen source, indicating that nitrate had little effect on the utilization of ammonia by the tissue. This suggests that ammonia is an intermediate

in the reduction of nitrate, a view for which further evidence is presented later.

The ability of the tissue to utilize alanine, glutamic acid, and aspartic acid in the presence of nitrate was tested with amino acid concentrations of 0.1 M and $5 \times 10^{-5}$ M (corresponding to points of maximum growth, fig. 1) and $10^{-3}$ M (corresponding to the point of minimum growth, fig. 1). In the series in which alanine was tested the media contained $8.36 \times 10^{-3}$ M nitrate with 14 atom % $N^{15}$ (Nos. C1, C3 and D1) as well as $8.36 \times 10^{-3}$ M $N^{15}$-labeled nitrate plus DL-alanine at concentrations of 0.1 M (Nos. C2 and D2), $10^{-3}$ M (No. D3), and $5 \times 10^{-5}$ M (Nos. C4 and D4). Numbers C1 and C3 are the controls for Nos. C2 and C4 respectively. A comparison of numbers C1 and C2 shows that when 0.1 M DL-alanine was present with nitrate, there was a 2.5-fold increase in percent nitrogen and a 9-fold decrease in $N^{15}$ concentration in the tissues. D1 and D2 showed similar results with a 3-fold increase in percent nitrogen and a 10-fold decrease in atom % $N^{15}$ excess. With the most dilute alanine concentration (Nos. C4 and D4, $5 \times 10^{-5}$ M) no marked change in percent nitrogen or $N^{15}$ concentration was noted, nor was any expected, since so little alanine was supplied. When alanine was supplied at $10^{-3}$ M concentration (No. D3) there was only a 1.2-fold increase in total nitrogen and a 1.7-fold decrease in $N^{15}$ concentration. These data show that at 0.1 M and $10^{-3}$ M concentrations of DL-alanine, the tissues could utilize both the nitrate and the amino acid. At $5 \times 10^{-5}$ M, DL-alanine was not present in high enough concentration to produce any marked dilution of the $N^{15}$. However, it did serve as a control to show that such low concentrations of alanine produced no marked effect on tissue nitrogen content or nitrate utilization.

The next series of experiments with L-glutamic acid was performed as the previous experiments with alanine. The media contained the following nitrogen sources: $8.36 \times 10^{-3}$ M nitrate with 14 atom % $N^{15}$ (Nos. E1 and F1, controls), $8.36 \times 10^{-3}$ M labeled nitrate plus 0.1 M unlabeled L-glutamate (Nos. E2 and F2), $10^{-3}$ M L-glutamate (No. F3), or $5 \times 10^{-5}$ M L-glutamate (Nos. E3 and F4). With 0.1 M glutamate in the medium the data showed a 1.4-fold increase in percent nitrogen with a 10-fold decrease in $N^{15}$ concentration with series E, and a 2-fold increase in percent nitrogen and an 11-fold decrease in $N^{15}$ concentration with series F. With $5 \times 10^{-5}$ M L-glutamate (Nos. E3 and F4), as with alanine at this concentration, no marked changes were noted. With $10^{-3}$ M L-glutamate (No. F3) there was no change in percent nitrogen but a 1.5-fold decrease in $N^{15}$ concentration. The data indicated that the tissues could utilize both the nitrate and the L-glutamate, as was the case with alanine, and that the glutamate was the preferred source of nitrogen.

With aspartic acid, again the control medium (Nos. G1 and H1) contained $8.36 \times 10^{-3}$ M nitrate (14 atom % $N^{15}$), while the other media contained in addition to the $N^{15}$-labeled nitrate, 0.1 M L-aspartate (Nos. G2 and H2), $10^{-3}$ M L-aspartate (No. H3), or $5 \times 10^{-5}$ M L-aspartate (Nos. G3 and H4). When 0.1 M aspartate was employed in number G2, a 1.8-fold increase in percent nitrogen and a 10-fold decrease in $N^{15}$ concentration was observed. With number H2, there was a 2.8-fold increase in percent nitrogen and a 9.4-fold decrease in $N^{15}$ concentration. Again with $5 \times 10^{-5}$ M amino acid (Nos. G3 and H4) there were no marked changes. With $10^{-3}$ M L-aspartate (No. H3), a 1.2-fold increase in percent nitrogen and a 1.7-fold decrease in $N^{15}$ concentration was observed. Here again the amino acid, L-aspartic acid, was used together with nitrate when both were present in the same medium, and again the amino acid was the nitrogen source of choice by the tissue.

Glycine, in contrast to alanine, glutamate, and aspartate, does not encourage growth at high concentrations (7), so glycine was supplied to determine whether responses of the tissues to glycine differed from those to alanine, glutamate, and aspartate. The control medium (No. I1) contained $8.36 \times 10^{-3}$ M nitrate (14 atom % $N^{15}$) and the other media contained glycine in addition at concentrations of 0.1 M (No. I2), $10^{-3}$ M (No. I3), or $5 \times 10^{-5}$ M (No. I4). When 0.1 M glycine was added to the medium (No. I2), a 2.1-fold increase in percent nitrogen and a 6-fold decrease in $N^{15}$ concentration were noted. At $10^{-3}$ M glycine concentration (No. I3) a 1.2-fold increase in percent nitrogen occurred accompanied by a 23-fold decrease in $N^{15}$ concentration, a decrease which contrasts with the 1.5 to 2.1-fold decreases noted with the other amino acids at $10^{-3}$ M. Apparently the tissues used $10^{-3}$ M glycine more readily than nitrate, alanine, glutamate, or aspartate. With the lowest glycine concentration used ($5 \times 10^{-5}$ M, No. I4), no marked changes were noted. In this series of experiments as in the previous three series, the tissue used the amino acid preferentially in the presence of nitrate.

Since the results of the experiments just described showed that the tissue cultures could utilize the amino acids supplied in the presence of nitrate, it appeared interesting to ascertain whether any difference arose in the distribution of $N^{15}$ in tissue amino acids when $N^{15}$-labeled amino acids were supplied at different concentrations with nitrate in the medium. Table III contains the data from such determinations of $N^{15}$ distributions. The tissue was incubated for eight hours in a medium containing unlabeled nitrate and $N^{15}$-labeled amino acid and analyzed by methods described earlier in this paper. The same type of experiment was carried out using $N^{15}$-labeled nitrate as the sole nitrogen source in order to obtain the $N^{15}$ distribution in amino acids when the tissue was grown on the basal medium for a short period.

$N^{15}$-enriched nitrate (14 atom % $N^{15}$) at a concentration of $8.36 \times 10^{-3}$ M was supplied as the sole nitrogen source to 205 gm of sunflower crown gall tissue. All components isolated from the tissue hydrolysate were found pure by chromatographic test except cystine, which contained some leucine, and serine and threonine, which slightly contaminated each other. "Ammonia" contained the highest $N^{15}$ con-

centration. The ammonia fraction isolated from the hydrolysate did not represent ammonia free in the tissue, but free ammonia plus large amounts of ammonia from decomposition of nitrogenous compounds during the acid hydrolysis. The value of 1.068 atom % $N^{15}$ for ammonia was undoubtedly lower than that of the free ammonia, for the lower $N^{15}$ concentration of all other compounds isolated indicates that any ammonia which they formed by decomposition during hydrolysis would dilute the $N^{15}$ of the free ammonia. The higher $N^{15}$ concentration in ammonia than in any other compound clearly indicated that nitrate was reduced via ammonia. The $N^{15}$ concentrations of the amino acids isolated differed over a 6-fold range. The $N^{15}$ concentration of glutamate, 0.314 atom % $N^{15}$ excess, was more than double that of any other amino acid isolated. Alanine, lysine, and glycine had relatively high $N^{15}$ concentrations.

In similar experiments, tissue cultures were supplied with $N^{15}$-labeled DL-alanine (7.5 atom % $N^{15}$) and unlabeled nitrate. In one such experiment alanine was supplied at $10^{-3}$ M concentration, the concentration corresponding to the point of minimum growth (fig. 1). The tissue (237 gm) was incubated eight hours in 300 ml of the basal medium plus alanine, and then was hydrolyzed and fractionated. All components isolated were pure by test on paper chromatograms except cystine, which contained leucine, and serine and threonine, which contaminated each other. The value for alanine is missing, but alanine doubtless contained a higher $N^{15}$ concentration than threonine, since $N^{15}$-labeled alanine was supplied in the medium. The fact that threonine contained a higher $N^{15}$ concentration (0.314) than did glutamic acid (0.238) is notable and should be verified before accepting its significance. Methionine (0.147) and serine (0.098) were higher than aspartic acid (0.092). The $N^{15}$ concentrations of the remaining components showed no pronounced grouping of compounds. "Ammonia" had a relatively low level of $N^{15}$ when the tissues were supplied $N^{15}$ in alanine in contrast to when they were supplied $N^{15}$ in nitrate. Nitrate assimilation apparently went by way of ammonia, but evidently nitrogen was transferred from alanine chiefly by transamination, a reaction which does not release ammonia.

A related experiment was performed with 150 gm of tissue in 200 ml of basal medium plus 0.1 M DL-alanine (7.5 atom % $N^{15}$). This concentration corresponded to the second maximum on the growth curve (fig. 1). The tissue was incubated eight hours in the medium and then was washed, hydrolyzed, and fractionated. Paper chromatography indicated that all the fractions isolated were pure except serine and threonine, which were mutually contaminated. Alanine contained the highest $N^{15}$ concentration (5.17) as it was supplied in the medium. Glutamic acid was next highest with 2.13 atom % $N^{15}$ excess. Threonine (0.814), "ammonia" (0.687), and aspartic acid (0.551) also were high. Here again threonine showed striking activity. Little significance can be attributed to the moderately high $N^{15}$ concentration in ammonia, for the origin of much of this ammonia is unknown. The remaining components contained smaller concentrations of $N^{15}$, and in contrast to the previous experiment with $10^{-3}$ M alanine, methionine contained a particularly small relative $N^{15}$ concentration.

In the first experiment with glutamic acid, the tissue (234 gm) was incubated for eight hours in 300 ml of the basal medium plus $10^{-3}$ M L-glutamic acid (7.5 atom % $N^{15}$). The amino acids were separated from the hydrolysate of the washed tissue; all were pure as judged by paper chromatography except valine, which contained xanthine; cystine, which contained leucine and cytosine; and serine and threonine, which showed some mutual contamination. Glutamic acid contained the highest $N^{15}$ concentration (0.563), as labeled glutamic acid was added to the medium. The next highest $N^{15}$ concentration was found in alanine (0.263). Other fractions containing high $N^{15}$ concentrations were methionine (0.134), aspartic acid (0.128), and threonine (0.109).

In the last experiment of this series, 218 gm of tissue was incubated eight hours in 200 ml of basal medium plus 0.1 M $N^{15}$-labeled L-glutamic acid (7.5 atom % $N^{15}$). Ethanolamine was contaminated with alanine and ammonia; valine contained xanthine; cystine contained leucine and cytosine; and serine and threonine had slight mutual contamination. The remaining components appeared pure by paper chromatography. As expected, glutamic acid had the highest $N^{15}$ concentration (3.46). In agreement with the experiment with $10^{-3}$ M glutamic acid, alanine (2.56) had the next highest $N^{15}$ concentration. The atom % $N^{15}$ excess for "ammonia" (1.15) was high relative to that found when $10^{-3}$ M glutamic acid was supplied the tissue; this high level of $N^{15}$ in "ammonia" also was noted when $N^{15}$-labeled alanine was supplied at 0.1 M concentration. Methionine contained 0.693 atom % $N^{15}$ excess, approximately the same as aspartic acid (0.663). The observed analysis of 0.563 atom % $N^{15}$ excess for ethanolamine is higher than the true value for ethanolamine, since this fraction was contaminated with ammonia and alanine, both of which contained high $N^{15}$ concentrations. Compounds other than these cited had substantially lower concentrations of $N^{15}$.

## Discussion

The data presented in this paper have brought to light several interesting aspects of the nitrogen metabolism of sunflower crown gall tissue cultures. When ammonia was the sole source of nitrogen for the tissue, it was utilized at a rate comparable to nitrate, although the ammonia was supplied at half the optimum concentration for nitrate. When nitrate and ammonia were offered simultaneously to the tissue, the nitrate had no effect on the utilization of ammonia as determined by $N^{15}$ analyses (table II). This indicated that ammonia was an intermediate in the reduction of nitrate. In another experiment (table III) in which $N^{15}$-labeled nitrate was the sole source of nitrogen, the tissue was fractionated after four hours'

incubation, and the ammonia isolated from the hydrolysate contained a significantly higher $N^{15}$ concentration than did any other component isolated, even though its concentration of $N^{15}$ was diluted by ammonia arising from decomposition of nitrogenous material during acid hydrolysis of the tissue. Ammonia has been demonstrated amply by C. C. Delwiche (1) and others as an intermediate in the reduction of nitrate in a variety of tissues from higher plants. Our experiments show that this is also the case in sunflower crown gall tissue cultures.

The results of the experiments in which tissues were grown for one week in media containing $N^{15}$-labeled nitrate as well as various concentrations of glutamic acid, aspartic acid, alanine, or glycine (table II) showed that the tissues did not utilize exclusively either the nitrate or amino acid but used both as nitrogen sources. When the amino acids were supplied at $5 \times 10^{-5}$ M concentration, corresponding to the first maximum on the growth curves (fig. 1), no large increases in percent nitrogen or decrease in $N^{15}$ concentration of the tissues were noted. Little change was expected, since the amino acid concentration was so small. Appreciable changes were noted, however, at higher amino acid concentrations, $10^{-3}$ M and 0.1 M, corresponding respectively to the point of minimum growth and the second point of maximum growth (fig. 1). Amino acids at these two concentrations always increased the percent total tissue nitrogen and decreased the tissue $N^{15}$ concentration relative to cultures on $N^{15}$-enriched nitrate without a supply of amino acid. These changes were most notable when 0.1 M amino acid was added to the medium (with the exception of $10^{-3}$ M vs. $10^{-1}$ M glycine). Summarizing the results when alanine, glutamate, aspartate and glycine were tested at 0.1 M concentration (amino acid to nitrate ratio of 12), there was a 2.2-fold average increase in percent nitrogen and a 9.3-fold average decrease in atom % $N^{15}$ excess relative to the nitrate cultures. With $10^{-3}$ M glutamate, aspartate and alanine (amino acid to nitrate ratio of 0.12), there was an average 1.1-fold increase in percent nitrogen and an average 1.8-fold decrease in $N^{15}$ concentration compared with the cultures limited to $N^{15}$-nitrate. When $10^{-3}$ M glycine was used, there was only a 1.2-fold increase in percent nitrogen but a 23-fold decrease in $N^{15}$ concentration; this was about 13 times the decrease in $N^{15}$ concentration obtained with alanine, glutamate, and aspartate at $10^{-3}$ M Apparently $10^{-3}$ M glycine competed much

TABLE III

UTILIZATION OF $N^{15}$-ENRICHED COMPOUNDS BY SUNFLOWER CROWN GALL TISSUE CULTURES

| COMPONENT ANALYZED | ATOM % $N^{15}$ EXCESS IN COMPONENT ANALYZED | | | | |
|---|---|---|---|---|---|
| | NITROGEN SOURCE ENRICHED WITH $N^{15}$ | | | | |
| | NITRATE * | 0.001 M ALANINE † | 0.1 M ALANINE † | 0.001 M GLUTAMIC ACID ‡ | 0.1 M GLUTAMIC ACID ‡ |
| Ammonia | 1.068 | 0.089 | 0.687 | 0.087 | 1.15 |
| Alanine | 0.148 | .... | 5.17 | 0.263 | 2.56 |
| Arginine | 0.095 | 0.056 | 0.273 | 0.058 | 0.139 |
| Aspartic acid | .... | 0.092 | 0.551 | 0.128 | 0.663 |
| Cystine | 0.047 (+ leucine) | 0.063 (+ leucine) | 0.415 | 0.050 (+ leucine, cytosine) | 0.128 (+ leucine, cytosine) |
| Ethanolamine | .... | 0.046 | .... | 0.054 | 0.563 (+ alanine, ammonia) |
| Glutamic acid | 0.314 | 0.238 | 2.13 | 0.563 | 3.46 |
| Glycine | 0.121 | 0.086 | 0.283 | 0.086 | 0.193 |
| Histidine | 0.017 | .... | 0.306 | 0.040 | 0.093 |
| Isoleucine | 0.098 | 0.065 | 0.428 | 0.084 | 0.146 |
| Leucine | 0.046 | 0.061 | 0.215 | 0.064 | 0.154 |
| Lysine | 0.135 | .... | 0.228 | 0.067 | 0.174 |
| Methionine | 0.050 | 0.147 | 0.225 | 0.134 | 0.693 |
| Phenylalanine | 0.056 | 0.076 | 0.205 | 0.098 | 0.273 |
| Proline | 0.082 | 0.083 | 0.226 | 0.050 | 0.163 |
| Serine | 0.066 (trace threonine) | 0.098 (trace threonine) | 0.350 (trace threonine) | 0.097 (trace threonine) | 0.311 (trace threonine) |
| Threonine | 0.056 (trace serine) | 0.314 (trace serine) | 0.814 (trace serine) | 0.109 (trace serine) | 0.190 (trace serine) |
| Tyrosine | 0.103 | 0.060 | .... | 0.065 | 0.113 |
| Valine | 0.072 | 0.072 | 0.308 | 0.059 (+ xanthine) | 0.173 (+ xanthine) |
| Humin | 0.953 | 0.061 | 0.195 | 0.036 | 0.135 |
| Hydrolysate | 0.198 | 0.157 | 1.44 | 0.114 | 0.853 |

* Tissue grown 4 hours in a medium containing 0.00836 M nitrate (14 atom % $N^{15}$) as sole nitrogen source.
† Tissue grown 8 hours in a medium containing 0.00836 M nitrate and 0.1 M or 0.001 M DL-alanine (7.5 atom % $N^{15}$) as mixed nitrogen source.
‡ Tissue grown 8 hours in a medium containing 0.00836 M nitrate and 0.1 M or 0.001 M DL-glutamate (7.5 atom % $N^{15}$) as mixed nitrogen source.

more effectively with nitrate as the source of nitrogen for the tissue than did glutamate, aspartate, or alanine.

At first it appears unusual that glycine should serve so well as a nitrogen source when supplied at $10^{-3}$ M concentration, since Riker and Gutsche (7) earlier reported that this concentration gave complete inhibition of growth of tissue cultures. Our data (table II) show that the low level of $N^{15}$ in the tissue was not a result of inability of the tissue to grow. A 24.8% increase in growth was obtained; the controls increased 23.5%. Thus, since the tissue grew and on analysis had an $N^{15}$ concentration one-twenty-third of that of the controls, it must be concluded that glycine was used preferentially to nitrate. The growth studies of Riker and Gutsche, which showed that twenty amino acids inhibited growth at about $10^{-3}$ M concentration, were carried out with solid agar media. The experiments with alanine were repeated in liquid media in shake flasks, and at $10^{-3}$ M alanine concentration, less inhibition was obtained in this liquid medium than when the solid medium was employed. Perhaps the amino acid in a liquid medium did not inhibit growth as completely as in the agar medium. Alternatively, the entire growth curve in the liquid medium might have been shifted along the X axis, so that some concentration other than $10^{-3}$ M represented the amino acid concentration at which maximum growth inhibition was obtained. Slower diffusion of nutrients and/or excreted metabolic products through an agar gel as compared to more rapid diffusion through a solution and differences in aeration between solid and liquid culture media may explain some of the variation between cultures grown on agar or in liquid.

The "flooding" of tissue with an amino acid which is not completely assimilated or removed by washing complicates interpretation of data on total nitrogen and the relative utilization of amino acid and nitrate from mixtures of the two. However, physical uptake of an amino acid by the cells could not have accounted for all the observed dilution of $N^{15}$ (table II), for the dilution of $N^{15}$ was proportionately greater than the increase in percent nitrogen, indicating a net uptake and utilization of the amino acid nitrogen. In addition, the experiments in which the tissues were incubated for only eight hours in a medium containing $N^{15}$-labeled alanine or glutamic acid showed that there was rapid incorporation of the $N^{15}$-labeled amino group into every amino acid isolated from the tissue hydrolysates. Moreover, the data on total nitrogen and alpha-amino nitrogen in table I show that when tissues were grown on agar media containing various concentrations of alanine, glutamic acid, or aspartic acid in the presence of nitrate, the supernatant fluid from homogenized tissue contained more total nitrogen than could be accounted for by the increases in free-amino nitrogen.

The distribution of $N^{15}$ among the amino acids isolated from tissue hydrolysates when $N^{15}$ was added in various forms was not unusual (table III). When tissue was incubated four hours in a medium containing $N^{15}$-enriched nitrate, glutamate contained a higher $N^{15}$ concentration than any other amino acid isolated. Glutamic acid has been shown to be the most active amino acid in many animal, plant and bacterial tissues. The high level of $N^{15}$ in ammonia suggests its formation as an intermediate in nitrate reduction.

When tissue was incubated eight hours in a medium containing $10^{-3}$ M $N^{15}$-labeled DL-alanine (table III), all of the amino acids isolated from the tissue hydrolysate contained $N^{15}$. Alanine no doubt had the highest atom % $N^{15}$ excess, as it was supplied in the medium, but the alanine sample was not analyzed. Otherwise, the highest $N^{15}$ concentration was in threonine, which was substantially higher than glutamic acid. This unusual result should be verified, for present concepts of plant tissue metabolism offer no path for the rapid conversion of either alanine or the alanine amino group to that of threonine except by way of glutamate. Methionine also was quite active; it contained about 50% greater $N^{15}$ concentration than serine and aspartic acid.

When 0.1 M DL-alanine was used (table III), some differences in labeling of the amino acids from those formed in the presence of $10^{-3}$ M DL-alanine were noted. Alanine, the amino acid supplied, contained the highest $N^{15}$ concentration. Glutamic acid was next highest, while threonine was third. However, threonine still contained a higher $N^{15}$ concentration than aspartic acid and serine. With $10^{-3}$ M alanine in the medium, methionine was fourth highest in $N^{15}$ concentration, but with 0.1 M alanine, methionine was third from the least active amino acid.

The results of the experiment with $10^{-3}$ M DL-glutamic acid (table III), reveal the high activity of alanine. The fact that alanine contains twice the $N^{15}$ concentration of aspartic acid is surprising, since the glutamic acid–aspartic acid transaminase was more active than the glutamic acid–alanine transaminase in preparations from sunflower tissue cultures (table I). However, as the experiments with the transaminases were carried out with a tissue suspension, and these tracer experiments utilized intact tissue, the results are not entirely comparable. Methionine contained a high $N^{15}$ concentration, as it did in the experiment with $10^{-3}$ M alanine. Aspartic acid was higher than threonine, although threonine was still more active than serine. Phenylalanine, which had about the same $N^{15}$ level as serine, contained a higher $N^{15}$ concentration than tyrosine, again suggesting conversion of phenylalanine to tyrosine by this tissue. Glycine again was only moderately active.

The concentration of $N^{15}$ in the amino acids from tissues supplied 0.1 M DL-glutamic acid (table III) decreased in this order: glutamic acid, alanine, methionine, aspartic acid, serine, phenylalanine, glycine and threonine. Alanine, methionine, and aspartic acid are in the same order as in the experiment with $10^{-3}$ M DL-glutamic acid. Methionine had only about a fourth the $N^{15}$ level of alanine, but about the same level as aspartic acid. Serine and glycine were higher than threonine in contrast to the experiment with $10^{-3}$ M glutamic acid.

The results obtained to date do not indicate a clear

explanation for the mechanism of the unusual growth response of sunflower tissue cultures to added alanine, glutamic acid, or aspartic acid. These amino acids are particularly active in transamination, but transaminase activities showed no apparent correlation with growth of tissue or with the level of amino acid supplied to growing tissues. Hence, there is no experimental evidence to support the idea that the effect of alanine, glutamic acid and aspartic acid on growth is directly a result of changing the transamination capacity of the tissue.

It is possible that the effect of amino acids is secondary, and that the keto acids formed by transamination are the primary agents inducing the growth responses. Hildebrandt and Riker (2, and unpublished data) showed that 0.5% pyruvate in the presence of 2% sucrose allowed poor growth of sunflower tissue, and lower concentrations of pyruvate allowed better growth; i.e., pyruvate induced a growth curve with one peak. Alanine in the same range of concentrations induced a growth curve with two peaks. These facts offer no support for the idea that the pyruvate arising from alanine is responsible for the observed effect of alanine on growth of the tissue.

The experiments described here establish the simultaneous utilization of nitrate and glutamate, aspartate, or alanine by sunflower crown gall tissue cultures at concentrations of amino acids which both induced and inhibited growth on solid media. Glycine, one of the seventeen amino acids which failed to induce the second growth maximum, also was used simultaneously with nitrate. At $10^{-3}$ M concentration, glycine was utilized readily and to a greater extent than glutamate, aspartate and alanine. This rapid utilization of glycine deserves further investigation. The relatively high activities of threonine, serine, methionine and phenylalanine have indicated no rational explanation of the growth effects, but have suggested that in intact tissue several transaminases may operate at rates comparable to the transaminases for glutamic acid, aspartic acid and alanine.

## Summary

Investigation of the nitrogen metabolism of sunflower crown gall tissue cultures has shown that:

1. Although the tissues contained active glutamic acid–aspartic acid and glutamic acid–alanine transaminases, there was no correlation between the activities of these enzymes in crude tissue homogenates and the reported growth of the tissue at several levels of glutamate, aspartate, or alanine supplied the tissue in the medium.

2. Nitrate had little effect on ammonia uptake by the tissues. Ammonia was an intermediate in the reduction of nitrate.

3. When nitrate was supplied together with alanine, glutamic acid, aspartic acid, or glycine, the tissue utilized both the nitrate and the amino acid.

4. When tissue was incubated for short periods in a medium containing $N^{15}$-labeled DL-alanine or DL-glutamic acid, the distribution of the $N^{15}$ among the amino acids isolated from a tissue hydrolysate differed somewhat with the amino acid and with the concentration at which it was supplied. In general, however, glutamic acid was the most active amino acid, and alanine, aspartic acid, threonine, serine, methionine and phenylalanine were also highly active.

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# AUXIN INACTIVATION AND ITS RELATION TO LEAF DROP CAUSED BY THE FUNGUS OMPHALIA FLAVIDA[1]

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*Omphalia flavida* Maubl. & Rang. is the agent of the most destructive disease of coffee in the American tropics. Even conservative estimates by field workers indicate that the damages caused by this fungus in several Central and South American countries amount to an annual loss in coffee production valued at several million dollars. *O. flavida* is a member of the family Agaricaceae of the Basidiomycetes. Comprehensive modern surveys of the biology and pathological importance of this fungus have been given by Buller (1), by Wellman (14) and most recently by Sequeira (9).

The disease is characterized by the development, on the leaves of coffee and a wide range of other host plants, of round or slightly ovoid spots, 0.4 to 1.0 cm in diameter (fig. 1). The number of lesions per leaf varies from 1 to well over 100. In general, the main pathogenic effect of the fungus is the severe premature defoliation which it causes.

It has frequently been reported that in most cases the attacked coffee leaves which have fallen bear a lesion at the base of the leaf blade close to the petiole or on the petiole, whereas the leaves that remain attached to the tree may have a large number of lesions but none at the base of the blade. In general, a single lesion located at the point where the leaf blade begins to widen is enough to cause the leaf to drop. This effect has been generally interpreted, without experimental proof, as the result of mechanical destruction of the vascular tissue of the leaf by the fungus.

Our preliminary work on experimental defoliation of coffee in the field and, for comparison, of Coleus in the greenhouse, indicates that the defoliation caused by this fungus cannot be explained simply as a result of mechanical injury to the leaf. Further investigation has shown that the inactivation of plant growth hormones by *O. flavida* may be offered as a possible explanation of the leaf drop caused by this fungus. It is the purpose of this paper to present the evidence obtained from several lines of investigation which have led to this interpretation of the mechanism of the main pathogenic effect of the fungus.

## EXPERIMENTAL RESULTS

EXPERIMENTAL DEFOLIATION: Extensive gross inoculations of coffee trees in plantations at Turrialba, Costa Rica, with the gemmae of *O. flavida* indicated that whenever the fungus caused a lesion at the base of a leaf, or on the petiole, the leaf always dropped within 5 days to 1 week from the time of inoculation.

[1] Received January 23, 1953.

[2] Parker Fellow of Harvard University.

[3] Society of Fellows of Harvard University.

Similar experiments on *Coleus Blumei* in the greenhouse at Harvard, in which a large number of leaves were inoculated at the base of the blade close to the petiole, showed that the fungus readily attacked the leaves of this plant and that many infected leaves began to fall within 3 days, defoliation being completed within 6 to 9 days. The mature leaves always fell off before the younger ones. In both coffee and Coleus our controls indicated that intact leaves remained attached to the plants for 3 weeks or more after all of the inoculated leaves had fallen.

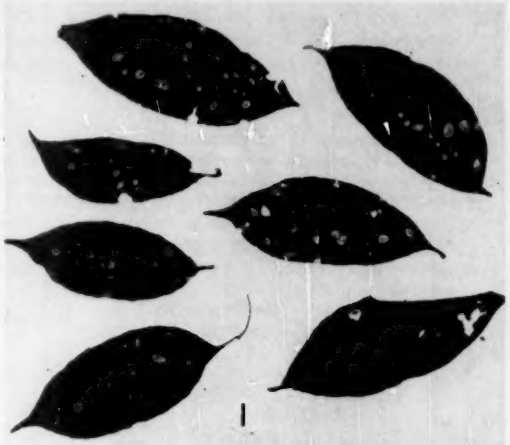


FIG. 1. Leaves of *Coffea arabica* L. collected at Turrialba, C. R., showing lesions resulting from the growth of *Omphalia flavida*.

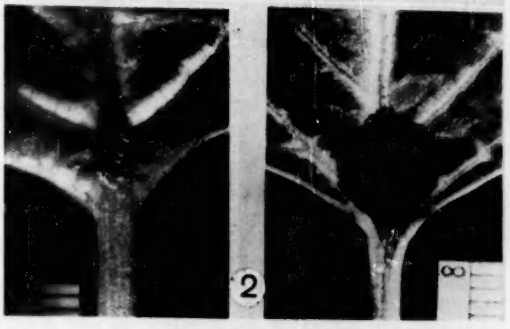
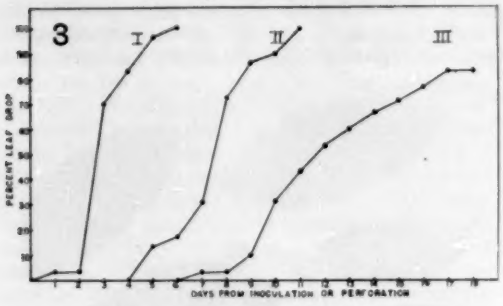


FIG. 2. Comparison of the extent of injury in Coleus leaves produced by the growth of *Omphalia flavida* (left) and by mechanical perforation (right). The fungus-infected leaf fell 5 days after inoculation. The perforated leaf was still attached 18 days after treatment. (Scale in millimeters.)

In order to determine the effect of mechanical injuries similar in extent and location to the Omphalia

lesions which cause shedding of coffee and Coleus leaves, a large number of leaves of these plants were perforated at the base of the leaf blade by the use of a paper-punch. Figure 2 compares a punched leaf of Coleus with a leaf bearing a fungus lesion which caused the leaf to drop. In all cases, both in coffee and in Coleus, the perforated leaves remained attached for a period considerably longer than that for the inoculated leaves. This is shown in figure 3 which represents the effect of these two treatments on groups of 30 Coleus leaves of comparable age. It is clear that no perforated leaves had fallen by the time all of the inoculated leaves had been shed, even though the perforation removed all, or almost all, of the vascular tissue and only a thin bridge of leaf tissue was left to provide a connection between the leaf blade and the petiole (fig. 2).



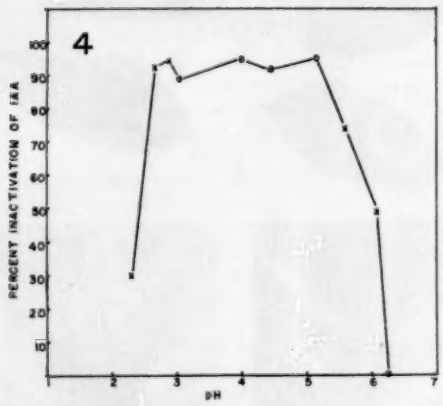




FIG. 3. Comparison of the rate of leaf abscission in Coleus induced by growth of *Omphalia flavida* at the base of the blade (I), growth by *Omphalia flavida* at the base of the blade with IAA-lanolin paste applied to the petiole below the lesion (II), and mechanical perforation at the base of the blade (III). In each group 30 leaves of comparable age were treated.

FIG. 4. The pH sensitivity of the auxin inactivation system in culture filtrate of *Omphalia flavida*. The curve combines the data of 2 separate experiments. The points obtained in each experiment are designated by a characteristic symbol.

The work of LaRue (6), Myers (7), Gardner and Cooper (3) and others has shown that when the leaf blades of Coleus are removed, the petioles begin to fall off within 3 days, the process of abscission being complete for all petioles very soon afterwards. We have repeated and confirmed these observations under our greenhouse conditions. Similarly, Miss Lucy Hastings, pathologist at the Inter-American Institute of Agricultural Sciences, Turrialba, Costa Rica, has reported to us that in coffee, drastically debladed leaves fall off much more rapidly than intact leaves. In one experiment more than 57% of the debladed leaves fell within 5 days as compared with 100% retention of the intact leaves at the end of the same period.

It would appear, therefore, that in the rapidity of the resulting abscission, the effect of the fungus lesion resembles the effect of complete removal of the leaf blade. Laibach (4, 5) has shown that orchid pollinia, and an auxin paste prepared from orchid pollinia, are able to retard leaf abscission in debladed leaves of Coleus, and LaRue (6), Myers (7), and Gardner and Cooper (3) have obtained similar results with synthetic growth hormones. Myers (7) has cited further evidence indicating that leaf abscission is normally retarded in intact leaves of Coleus by plant growth hormones which diffuse down from the leaf blade. He has also demonstrated that these substances can move through the cells of the petiole even when the vascular system is severed. In a study of the abscission of leaflets of bean, Shoji, Addicott and Swets (10) have shown a correlation between a fall of the auxin concentration in the leaflet and the approach of abscission, and have indicated that an auxin gradient across the abscission zone, rather than the absolute amount of auxin present, controls abscission. Wetmore and Jacobs (16), working with Coleus, have concluded that the diffusible auxin from the leaf blade is the factor which normally controls the pattern of leaf abscission; but they favor the interpretation that auxin exerts an indirect effect upon the abscission layer.

We have been able to show that if an additional source of growth hormone in the form of a 0.5% indoleacetic acid in lanolin is applied to the petioles of leaves inoculated with *O. flavida*, below the lesion caused by the fungus, leaf drop can be delayed considerably. This is shown in figure 2, which represents the effect of this treatment on 30 Coleus leaves. In many cases the petioles of leaves treated in this way remained attached for some time after the leaf blades had dropped off as the result of extended decay in the region of the fungus lesion.

These preliminary experiments on defoliation clearly suggested that *O. flavida* caused leaf drop by interrupting, in other than a purely mechanical way, the normal flow of auxin from the leaf blade to the petiole.

AUXIN INACTIVATION IN VITRO BY OMPHALIA FLAVIDA: Two possibilities for preventing the passage of auxin to the petiole immediately suggested themselves: 1. that the fungus utilizes the growth hormone as a substrate and therefore metabolically destroys it and 2. that the fungus produces a substance which

in some way inactivates the auxin thereby preventing it from inhibiting the abscission process. We shall describe the experiments which we have performed in attempting to determine if either of these possibilities is indeed the correct explanation.

All experiments were carried out with strain $T_4$ of *O. flavida*, originally isolated from lesions on leaves of *Coffea excelsa* growing at Atirro (Turrialba), Costa Rica (9). The fungus was cultured in all experiments on a synthetic liquid medium which previous nutritional experiments (9) had indicated to be suitable for good growth. This medium contained:

| | |
|---|---|
| Glucose | 20.0 gm |
| Ammonium tartrate | 9.2 gm |
| Potassium dihydrogen phosphate | 5.8 gm |
| Magnesium sulfate | 2.5 gm |
| Ferric chloride (1% sol.) | 0.6 ml |
| Thiamine hydrochloride | 100 μg |
| Distilled water | 1000 ml |

Each culture consisted of 40 ml of this medium in a 250-ml Erlenmeyer flask, inoculated with a number of gemmae of *O. flavida*, collected from young potato-dextrose agar cultures. The cultures were grown at room temperature for a period of 10 to 12 days on a reciprocating shake machine, at approximately 75 cycles per minute. The growth of the fungus under these conditions produces numerous spherical masses of mycelium dispersed in the medium.

In all experiments testing the capacity of the fungus to utilize growth hormones metabolically, or to inactivate them, synthetic indoleacetic acid (IAA) was used as the substrate. The determination of growth hormone in test solutions was carried out by means of the standard Avena test as described by Went and Thimann (15). On each occasion the curvature of a sensitivity standard of known concentration (25 μg IAA per l of agar) was determined, and these standards were used as a basis for the corrections necessary in comparing data from different experiments and in calculating absolute concentrations of growth substance in test solutions.

At the outset of these experiments it was shown (table I) that neither the culture medium (column A) nor a filtrate of medium in which fungus has grown (column B) possess any significant growth promoting activity. In order to test for auxin inactivation by fungus growth, IAA was added to the medium before inoculation to give a concentration of 250 μg/l. When this culture filtrate was tested for growth hormone activity 10 days after inoculation it gave no significant curvature (column D, table I), indicating a complete disappearance of the IAA. If IAA in the same concentration was maintained for 10 days in the liquid medium under the same conditions but not inoculated with *Omphalia flavida*, the medium showed a high degree of activity at the end of this period, as is shown in column C of table I.

Further experiments not reported in detail have indicated that the inactivation of IAA by the fungus probably occurs within 2 to 3 days. The growth of the fungus for a period of 10 days changes the pH of the medium from approximately 5.0 to approximately 3.75. It was shown that this increase of acidity is not responsible for the inactivation by adjusting medium containing IAA to pH 3.5 with 1 *N* HCl, autoclaving and allowing to stand on the shake machine for 5 days. A test at the end of this time showed no loss of auxin activity.

In order to determine the effect of metabolic products of the fungus on IAA in the absence of the fungus, the mycelia of 12-day-old cultures were carefully filtered out; to the remaining liquid IAA was added, and the mixture was incubated at room temperature for 1 hour in most cases. At the end of this period the preparation was heated to 95–100°C for 10 minutes, or autoclaved at 15 pounds pressure for an equal length of time to inactivate the enzymes.

TABLE I

AUXIN CONTENT, EXPRESSED IN DEGREES OF CURVATURE OF AVENA PLANTS, OF CULTURE MEDIUM, CULTURE MEDIUM IN WHICH *O. flavida* HAS GROWN FOR 10 DAYS (CULTURE FILTRATE), CULTURE MEDIUM CONTAINING 250 μG/L IAA,* CULTURE MEDIUM CONTAINING 250 μG/L IAA IN WHICH *O. flavida* HAS GROWN FOR 10 DAYS

| TEST NO. | CULTURE MEDIUM (A) | CULTURE FILTRATE (B) | CULTURE MEDIUM WITH IAA ADDED (C) | CULTURE FILTRATE WITH IAA ADDED (D) |
|---|---|---|---|---|
| 1 | 0.0 ± 0.0 | 1.0 ± 0.5 | 23.3 ± 2.1 | 1.1 ± 0.4 |
| 2 | 0.6 ± 0.3 | 0.5 ± 0.3 | 21.2 ± 1.3 | 0.9 ± 0.5 |
| 3 | 0.7 ± 0.4 | 0.7 ± 0.4 | 21.0 ± 1.3 | 0.3 ± 0.2 |
| 4 | 0.9 ± 0.5 | 3.3 ± 1.2 | 23.6 ± 1.5 | 0.8 ± 0.3 |
| 5 | 0.9 ± 0.3 | 1.9 ± 0.7 | 25.5 ± 2.4 | 1.5 ± 0.5 |
| 6 | ..... | ..... | 35.2 ± 2.5 | 0.4 ± 0.3 |
| 7 | ..... | ..... | ..... | 0.7 ± 0.5 |
| Average † | 0.6 | 1.5 | 26.1 | 0.8 |

* The concentration in the test blocks used in the Avena test is one-half that of the original solution because of dilution with 3% agar in the preparation of the blocks.

† In computing averages all curvatures were corrected on the basis of a sensitivity standard of 12.0 degrees.

The results, shown in column A of table II, indicate that the culture filtrate can inactivate up to 500 μg/l of indoleacetic acid completely in the time allotted. The small remaining amounts of auxin in the table are not significant, since concentrations of less than 10 μg/l (5 μg/l in final test dilution) are below the effective sensitivity of the Avena test.

The cytoplasmic contents of the mycelium of *O. flavida* show the same auxin-inactivating properties as those described for the culture filtrates. A water extract of carefully washed mycelium, ground in a Waring microblender, was as effective as the culture filtrates in the inactivation of IAA. When IAA (50 μg/l) was added to the mycelium extract and this mixture was assayed for auxin activity at the end of 1 hour, a remaining concentration of 3 μg/l was found.

The inactivation of IAA by Omphalia culture filtrate can be completely prevented if this filtrate is

TABLE II

INACTIVATION OF IAA BY CULTURE FILTRATE OF *O. flavida* AND THE EFFECT OF HEAT UPON THIS INACTIVATION. THE INCUBATION PERIOD IS ONE HOUR EXCEPT WHERE NOTED

| TEST NO. | CONCENTRATION OF ADDED IAA IN μG/L | CONCENTRATION OF IAA IN μG/L | |
|---|---|---|---|
| | | FILTRATE INCUBATED WITH IAA | HEATED FILTRATE INCUBATED WITH IAA |
| | | A | B |
| 1 | 50 | 6 | 54 |
| 2 | 50 | 4 | 57 |
| 3 | 50 | 3 | 50 |
| 4 | 50 | 7 | ... |
| 5 | 50 | 7 | ... |
| 6 | 250 * | 1 | 146 † |
| 7 | 250 | 3 | 78 † |
| 8 | 250 | 2 | ... |
| 9 | 250 * | 7 | ... |
| 10 | 500 | 7 | 270 † |

* Incubation period twelve hours.
† Represents maximum or nearly maximum curvature of Avena plants for the day and therefore does not necessarily indicate partial inactivation.

heated to 95–100°C for 10 minutes or autoclaved at 15 pounds pressure for an equal length of time, before adding the growth hormone. Some typical results of this treatment are shown in column B of table II and may be compared with the results obtained from unheated filtrate (column A) on each occasion.

Culture filtrates dialyzed in cellophane tubing for 18 hours, under constant agitation, are as effective as the undialyzed filtrates in the inactivation of IAA. For example, in two experiments in which IAA at an initial concentration of 50 μg/l was incubated for 1 hour with dialyzed fungus filtrate, the final concentrations measured were 5 μg/l and 2 μg/l, indicating essentially complete inactivation.

It seemed desirable to determine whether this heat-labile, nondialyzable system is pH sensitive. Inactivation tests, using 2-hour incubation periods, were carried out in a pH range of 2.3 to 7.2 in citric acid–disodium phosphate buffers. In each test equal volumes of dialyzed culture filtrate and buffer of the desired pH were mixed and IAA was added to a final concentration of 250 μg/l. The actual pH was then determined. The results of these studies are shown in figure 4. While it is not possible to determine a pH optimum for the system, it is clear that the system is pH sensitive, showing a high degree of activity between pH 2.65 and pH 5.60, and a rather striking decrease of activity on either side of these values.

EFFECT OF ENZYME INHIBITORS: Since the foregoing experiments have strongly suggested that the auxin-inactivating system in Omphalia culture filtrate is enzymatic in nature, an attempt was made to obtain a preliminary characterization of the system by testing the effects of several enzyme inhibitors upon the ability of the system to inactivate IAA. The results of these experiments are shown in table III in which the percentage of the original IAA concentration which was inactivated in the presence of the inhibitor is shown. In each experiment the inhibitor was added to the culture filtrate before the addition of IAA. Some difficulty was encountered in these experiments because of an unexplained variation in auxin-inactivating ability between different batches of culture filtrate. It was consequently difficult to select suitable concentrations of IAA or of inhibitor to show the effect of the inhibitor to best advantage. In addition certain of the inhibitors exerted a depressing effect upon the curvature of the Avena plants.

The most striking inhibition effect noted in these experiments was that produced by the reducing agents ascorbic acid and cysteine at a concentration of 1 gram per liter (table III, exps. 2, 3, and 4). These two substances afforded essentially complete protection for IAA and suggest that an oxidative enzyme is responsible for auxin inactivation by the culture filtrate. Moreover, if nitrogen gas is bubbled through the reaction mixture during the incubation period, the destruction of the growth hormone is considerably reduced (table III, exps. 5 and 6).

Potassium cyanide gives a rather variable degree of inhibition of the enzyme, but seems to afford at least partial protection to the growth substance. The results are somewhat complicated by the fact that potassium cyanide reduces the curvature of the Avena test even though ferrous sulfate is added to the reaction mixture after incubation to remove excess cyanide ions. Hydroxylamine also partially inhibits the activity of the enzyme, but is somewhat harmful to the Avena plants so that its full effectiveness cannot be evaluated.

These preliminary experiments with enzyme inhibitors would seem to indicate that auxin inactivation by Omphalia culture filtrate is carried out by an

TABLE III

THE INFLUENCE OF SEVERAL ENZYME INHIBITORS ON THE INACTIVATION OF IAA BY CULTURE FILTRATE OF *O. flavida*. THE INCUBATION PERIOD IS ONE HOUR EXCEPT WHERE NOTED

| TEST NO. | INHIBITOR | CONCENTRATION OF IAA IN μG/L | PERCENTAGE INACTIVATION OF IAA |
|---|---|---|---|
| 1 | None | 50–500 | ca. 100 * |
| 2 | Ascorbic acid 1 gm/l | 50 | 0.0 |
| 3 | Cysteine 1 gm/l | 50 | 2.6 |
| 4 | Cysteine 1 gm/l | 50 | 0.0 |
| 5 | $N_2$ † | 250 | 36.4 |
| 6 | $N_2$ † | 250 | 40.9 |
| 7 | KCN .005 M | 50 | 60.0 |
| 8 | KCN .01 M | 50 | 76.8 |
| 9 | KCN .01 M | 250 ‡ | 24.5 |
| 10 | $NH_2OH \cdot HCl$ .005 M | 50 | 45.0 |
| 11 | $NH_2OH \cdot HCl$ .01 M | 250 ‡ | 51.0 |

* Based on the experiments recorded in Table II.
† Water pumped nitrogen, 99.6% $N_2$.
‡ Incubation period two hours.

oxidative enzyme system which is only partly inhibited by cyanide and hydroxylamine.

## Discussion

The inability of mechanical injury to duplicate the abscission-promoting effect of *Omphalia flavida* and the effectiveness of added growth hormone in preventing abscission of infected leaves of Coleus strongly suggest that the fungus prevents the normal flow of auxin from the blade into the petiole by other than mechanical means. An experimental demonstration of the ability of the fungus grown *in vitro* to inactivate IAA has been given. A similar action of the fungus in the leaf of the host would provide a reasonable explanation of its abscission-promoting properties, and it is suggested that this is the mechanism of the main pathogenic effect of the fungus.

Several attempts were made to provide further evidence for this conclusion by demonstrating auxin inactivation by *O. flavida* in Coleus leaves in comparative diffusions from infected and uninfected leaves. The results of these experiments were inconclusive, however, because the yields of auxin obtained even from uninfected leaves were below the effective sensitivity of the Avena test. Myers (7) had obtained similar low yields from Coleus leaves at certain seasons of the year. Since such low auxin yields are sufficient to retard abscission of Coleus leaves, a direct demonstration that *O. flavida* does promote abscission by inactivating auxin in the leaf will be difficult to obtain.

Previous work on the relationship between fungi and plant hormones has dealt, as far as we are aware, with the production of auxin by these organisms, or with the response of the organisms to added auxin, rather than with auxin inactivation. It has been shown that numerous fungi and bacteria produce auxin in culture (see review by Went and Thimann (15), pp. 71–72). Recently Wolf (17) has identified IAA as a metabolic product of *Ustilago zeae* and has correlated its production with the tumor formation in the host induced by the fungus. Synthetic growth hormones are also reported to produce both stimulatory and inhibitory effects upon the growth of various microorganisms, the specific response depending upon the organism tested and the concentration used (8).

Since the filtrate of a culture in which *O. flavida* has grown is capable of inactivating added IAA, it is clear that the auxin is not directly metabolized by the fungus but rather that the organism produces and releases into the substrate, a substance which is capable of inactivating the auxin. The substance is also present within the cells of the fungus and we have not established whether it is actively secreted into the medium or is released through the process of autolysis.

Since the IAA inactivating substance is heat-labile, non-dialyzable, and pH-sensitive, we have concluded that it is an enzyme. Its pronounced inhibition by the reducing agents ascorbic acid and cysteine, and also by a nitrogen atmosphere strongly suggest that it is an oxidative enzyme. The sensitivity of the enzyme to the inhibitors potassium cyanide and hydroxylamine, which seems reasonably well established, might suggest that the enzyme involved is a peroxidase, but the pyrogallol test of Sumner and Gjessing (11) showed no peroxidase activity in the culture filtrate. Since pyrogallol is not oxidized by the culture filtrate, it is unlikely that the enzyme present is a polyphenol oxidase.

Auxin-inactivating enzymes have thus far been described only for the higher plants. There have been many reports of enzymatic auxin inactivation at cut surfaces and in crushed tissue extracts, but relatively few thorough studies of the actual systems involved. Tang and Bonner (12) have described an "indoleacetic acid oxidase" from etiolated seedlings of pea which they believe to be an iron-containing protein. Wagenknecht and Burris (13), on the other hand, have concluded that this same enzyme is a copper-containing protein, and they have reported a similar enzyme from the root sap of beans. The exact nature of the enzyme is apparently still in dispute. More recently Galston (2), in connection with an investigation of the biological significance of riboflavin, has re-examined Tang and Bonner's "indoleacetic acid oxidase" and believes it to be composed not of a single enzyme but of a light activated flavoprotein enzyme and a peroxidase. The flavoprotein is believed to produce hydrogen peroxide which is then utilized by the peroxidase in the oxidation of IAA.[4] It was considered likely that IAA is also a substrate for the flavoprotein.

Our study of the auxin-inactivating enzyme produced by *O. flavida* is not sufficient to permit any detailed comparison with the enzymes described from higher plants. It may be noted, however, that the pH sensitivity range of 2.65–5.60 for the inactivating system of the fungus differs from the sharp optimum between 6.2 and 6.7 described by Tang and Bonner (12) and 6.0 and 6.5 described by Wagenknecht and Burris (13). It seems unlikely that it could be a system of the sort described by Galston (2) because we were unable to obtain any evidence of peroxidase activity in the culture filtrate. It is not unreasonable to expect the enzyme system of *O. flavida* to differ from those of the higher plants in view of the vastly different relationships of the source organisms and the very different mode of action. Work is now in progress to obtain a more definite characterization of the enzyme involved.

## Summary

*Omphalia flavida* exerts its pathogenic effect on coffee and other hosts by bringing about severe premature defoliation. In many cases rapid leaf drop results from the presence of a single lesion at the base of the blade or on the petiole. Mechanical injury of comparable extent does not accelerate leaf abscission in coffee or in Coleus to the same degree. Abscission of Coleus leaves inoculated with the fungus can be

[4] In a further report, published since the present paper was submitted (Galston, A. W., J. Bonner and R. S. Baker, Arch. Biochem. 42: 456–470. 1953), this interpretation has been extended and confirmed.

delayed by application of synthetic IAA to the petiole below the lesion. This evidence suggests that the fungus accelerates abscission by preventing the normal flow of auxin from the blade to the petiole.

*O. flavida* inactivates IAA included in its culture medium; and culture filtrate, in the absence of mycelium, has been found to inactivate up to 500 μg/l of IAA in an hour. Extracts of ground mycelium possess the same auxin-inactivating properties.

Preliminary studies on the auxin-inactivating substance from Omphalia show that it is heat-labile, non-dialyzable, and pH-sensitive. It is strongly inhibited by ascorbic acid, cysteine, and a nitrogen atmosphere, and appears to be somewhat sensitive to potassium cyanide and hydroxylamine. It is concluded that it is an oxidative enzyme, but probably not a peroxidase or polyphenol oxidase. It is not clear whether the enzyme is actively secreted or is released by autolysis.

It is suggested that this auxin-inactivating enzyme is responsible for the main pathogenic effect of *Omphalia flavida*.

The authors wish to express their appreciation to Professor W. H. Weston, Jr., and Professor K. V. Thimann for their advice and encouragement during the investigation and for their valuable criticism of the manuscript, and to Professor R. H. Wetmore for many helpful suggestions and for making available unpublished data relating to leaf abscission. They are also indebted to Miss M. P. F. Marsden and to Miss Jane Richards for their assistance with the auxin assays.

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# DIRECT CHEMICAL PROOF OF ETHYLENE PRODUCTION BY DETACHED LEAVES[1]

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Ethylene production by leaves has long been suspected. Using potato leaf epinasty as a qualitative bioassay, Denny and Miller (3) showed production of an active emanation, presumably ethylene, by detached leaves of dandelion, rhubarb, calla, hollyhock and leafy stalks of peony. This work was extended by Denny (2), using chopped leaves of rhubarb, lettuce, potato, tomato, onion, Virginia creeper and rose. Nelson and Harvey (8) showed that chopped stalks and leaves of Golden Self-Blanching celery caused tomato leaf epinasty, while those of Winter Queen (a green celery) did not. Hall (5) used a non-specific chemical test as evidence for ethylene production by healthy detached leaves of rose and cotton.

Similar studies have been made with leaves affected by various agents. Williamson (13) showed that various kinds of leaves suffering from mechanical injury and leaves infected with various fungi produced appreciably more active emanation than did healthy intact leaves. Ross and Williamson (11) got similar results with leaves from virus-infected plants and with leaves injured by phytotoxic chemicals. Jackson (7) and Hall (6) observed increased production of ethylene by cotton leaves treated with defoliating agents. Gawadi and Avery (4), after confusing ethylene and ethylene chlorohydrin, suggested that ethyl-

[1] Received March 3, 1953.

ene may play a part in natural leaf abscission, but no data were presented. The rapid yellow coloration and early abscission of infected leaves may be correlated with an increased production of ethylene by the diseased leaves (13).

Because of the renewed interest in ethylene production by plant material other than fruits, and because the only claimed chemical determinations of ethylene production by leaves are non-specific (5, 6), it was of interest to prove ethylene production using a specific technique. Hence ethylene production by leaves of milk thistle (*Silybum marianum* Gaertn.) was demonstrated by accumulation of ethylene in mercuric perchlorate solution and its subsequent release to form the ethylene dibromide and N,N′-diphenylethylenediamine derivatives; the melting point of the latter served to identify ethylene gas.

It should be emphasized that all these studies have been made with leaves removed from the plant; hence all may be considered to be dying, although of varying degrees of original health. Ethylene production in healthy intact plants has not yet been shown, although preliminary studies by Rossetter and Jacobs (12) suggest this possibility.

## Preliminary Experiments

Trial runs were made using the triple response of etiolated pea seedlings (10) as a bioassay for ethylene production by leaves. Traces of ethylene were indicated from samples of tomato and thistle leaves, the latter appearing to produce a larger quantity. Another trial, with leaves of tomato and chard, was conducted using a flowing air system and the quantitative mercuric perchlorate analytical method described previously (15). With this technique, no ethylene was recovered from tomato leaves and only a trace from chard.

Since the amount of ethylene being produced appeared to be too small to recover from the necessarily large volume of air in a flowing air system, a static system was tried as described below. Using this method, an amount of ethylene was recovered from thistle leaves which suggested that it would be reasonably possible to accumulate enough for a suitable derivative and melting point determination.

Final determination of ethylene was accomplished using fresh green leaves of milk thistle; old, yellow leaves were avoided. During the experiments, the leaves turned yellow, and some became dry and brown, but no fungus or bacterial decay was evident. Thistle leaves were used because this plant appeared to produce as much or more ethylene than did tomato or chard leaves, and this thistle was much more plentiful at the time of year when this project was being carried out.

The apparatus used for accumulating the ethylene produced by leaves was adapted from the static system of measuring respiration as modified by Platenius (9). It consisted of a large tissue chamber, an oxygen supply bottle, and a Mariotte bottle to maintain constant pressure in the system (fig. 1). The tissue chamber (A) was a large Pyrex cylindrical jar (12″ × 24″) closed with a rubber gasket and a bolted-down steel plate (1). One liter of two normal potassium hydroxide was placed in the bottom of the chamber to absorb the carbon dioxide of respiration and a few drops of bromthymol blue indicator were added to verify the adequacy of the alkali throughout the experiment. The leaves were supported above the alkali on a heavy screen. Oxygen was supplied through a capillary from a five-gallon bottle (B). The Mariotte bottle (C) supplied the water for oxygen displacement, and kept the pressure in the system constant; it consisted of a five-gallon bottle with a hole bored in the bottom and a capillary air supply tube in the top. By the use of this system, ethylene accumulates in the atmosphere of the tissue chamber, but the oxygen and carbon dioxide concentrations remain essentially unchanged. Twelve such assemblies were

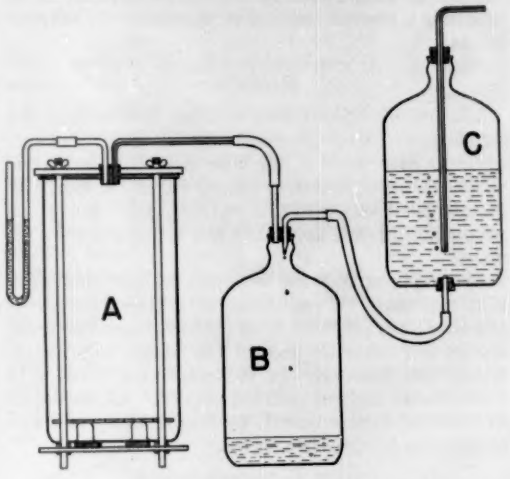


Fig. 1. Apparatus for accumulation of ethylene produced by plant tissue.

used simultaneously in each run, and they were maintained at 25°C in a constant temperature room.

At the end of each experimental period, the atmospheres of the twelve jars were combined by successively forcing them by water displacement through the sintered bubbler assembly previously reported (15). The graduated tube contained 35 ml of a solution of mercuric perchlorate (0.25 M in 2.0 M perchloric acid) and a few drops of *n*-butyl alcohol. This assembly was held at 0°C in an ice-filled Dewar flask.

Two full-scale runs were made; when the displacement of the atmospheres after each run was completed, the absorbent solution was made to 50 ml with additional mercuric perchlorate and two 2-ml portions were withdrawn for manometric quantitative analysis by the method of Young, Pratt, and Biale (15). The remaining solutions from the two runs were combined for preparation of the derivatives.

Preparation of the ethylene dibromide and N,N′-diphenylethylenediamine derivatives were accomplished with the technique and apparatus described previously

(14), the only difference being that the apparatus was evacuated, and then the ethylene-containing mercuric perchlorate solution was drawn in. This procedure avoided possible losses by dissociation during evacuation of the system.

The following controls were run: As the tissue jars were set up, they were flushed with air drawn from a compressor with an intake well above the roof of the laboratory; at the same time, a volume of air equal to that of the twelve tissue chambers was drawn from the same source and passed through mercuric perchlorate solution. This solution was then tested manometrically to demonstrate freedom from ethylene. Similarly, a volume of oxygen equal to that used in the experiments was drawn from the same oxygen pressure cylinder and tested for purity. All solvents and reagents were freshly distilled or examined for residue by evaporation of a suitable aliquot. In no case was a possible contaminating amount of ethylene located.

## Results

Manometric quantitative analysis showed that 13.5 kg of thistle leaves produced 10.8 ml (S.T.P.) of ethylene in four days in the first run. In the second run, 13.1 kg of leaves produced 6.0 ml of ethylene. Hence the total available was 16.8 ml; during the production of this amount, 400 l of oxygen were respired.

Melting points were obtained of the derivative N,N′-diphenylethylenediamine, of a known sample of this substance prepared from pure ethylene dibromide, and of a mixture of these. The values were determined simultaneously on the aluminum block of a Fisher-Johns melting point apparatus. All melted at 64.0–65.0°C (uncorrected), confirming the production of ethylene by detached leaves.

## Summary

Using methods reported previously, ethylene was identified as a product of detached leaves of milk thistle (*Silybum marianum* Gaertn.).

Technical assistance by Mr. Carl Tucker, Mr. Milton Workman, and Mr. Fred D. Howard is gratefully acknowledged.

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# METABOLISM OF SUCROSE AND RELATED OLIGOSACCHARIDES BY SPORES OF THE FUNGUS MYROTHECIUM VERRUCARIA[1]

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In an earlier study of invertase in spores of the fungus *Myrothecium verrucaria* it was postulated that metabolism of sucrose did not proceed through a hydrolytic pathway in spite of the presence of an excess of invertase (9). This was inferred from the equivalence of the measured invertase activity of living, metabolizing spores with that of killed spores. The present paper reports further studies related to the proposed non-hydrolytic mechanism. Experiments with the related sugars raffinose and melezitose and with turanose have also been carried out to compare the metabolic patterns with the structural relations of the sugars.

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## Methods

Spores of the fungus *Myrothecium verrucaria* (QM 460) were obtained from cultures grown on a medium composed of distilled water—1000 ml, $NH_4NO_3$—3.0 gm, $MgSO_4 \cdot 7\,H_2O$—2.22 gm, $KH_2PO_4$—2.59 gm, $K_2HPO_4$—2.21 gm, Agar—15 gm. A sheet of filter paper served as carbon source (9). Washed suspensions of spores in 0.05 M $KHPO_4$ buffer at pH 6.25 were used in all work unless otherwise specified.

Respiration measurements were by conventional Warburg techniques at 30°C using 1.0 ml of spore suspension in the main compartment, 0.5 ml of substrate—usually 3%—in the side arm, and 0.2 ml 10% KOH in the center well. Rates of respiration are given as $Q_{O_2}$ on a dry weight basis, i.e., $\mu$l $O_2$ per mg spores per hr or as $\mu$g sugar oxidized per mg spores per hr. To calculate this latter value a respiratory quotient of 1 was used. Actually, the R.Q. in sugar solutions increases from about 1.0 to about 1.2 after several hours. Thus the calculated quantities of sugar respired represent minimum values. Respiration of sugars in phosphate buffer is not significantly different from that in inorganic nutrient solution.

Carbohydrase activity was measured by determination of reducing sugars formed in suspensions incubated at 30°C on a reciprocal shaker with the dinitrosalicylic acid method of Sumner (14), or by specific determination of glucose using the glucose oxidase method of Keilin and Hartree (7). In all cases, rates of hydrolysis are reported in terms of $\mu$g reducing sugar (RS) formed per mg dry weight of spores per hour.

Total sugars were determined by hydrolysis with 2.5 *N* HCl at 60° for 10 minutes. After neutralizing with alkali, reducing sugars were determined as indicated above.

Assimilation is given as the increase in dry weight in $\mu$g per mg original dry weight per hr after incubation at 30°C on a reciprocal shaker. Aliquots of the suspensions were filtered through sintered glass crucibles of fine porosity, dried at 80°C, and weighed. Assimilation of sucrose by these spores is only slightly affected over a period of five hours by the inorganic composition of the medium, the rate being increased about 3% in phosphate buffer (pH 6.25), and about 9% in inorganic nutrient solution over that in distilled water.

Acid treatment of spores was effected by combining a suspension in distilled water with an equal volume of 0.2 *N* HCl, incubating at 30°C for twenty minutes, centrifuging, washing, and resuspending in buffer. Control samples received identical treatment except that equal quantities of distilled water were used instead of acid.

In any one experiment spores were obtained from cultures all of the same age. Unfortunately it was not practical to use spores of the same age in all experiments—most of the work being done with spores from cultures 8–30 days old. To a large extent the variations in absolute values reported in different tables can probably be ascribed to differences in spore age.

Table I

Effect of Azide on Metabolism of Sucrose

| | | Sugar metabolized or hydrolyzed $\mu$G/MG × HR | |
|---|---|---|---|
| | | $-NaN_3$ | $+NaN_3$ ($5 \times 10^{-4}$ M) |
| Respiration | (1) | 24 | 0 |
| Assimilation | (2) | 70 | 14 |
| Sugar metabolized | (1+2) | 84 | 14 |
| Invertase activity | ..... | 389 | 389 |

(Duration of experiment 4 hours; 6.5 mg spores/ml; inorganic nutrient solution.)

Table II

Effect of Method of Blocking Metabolism on Hydrolysis of Sucrose and Raffinose by M. verrucaria Spores

| Treatment | Rate of hydrolysis * | | Relative hydrolysis | |
|---|---|---|---|---|
| | Sucrose | Raffinose | Sucrose | Raffinose |
| Control ..... | 307 | 30 | 1.00 | 1.00 |
| Azide ($5 \times 10^{-4}$ M) .. | 307 | 67 | 1.00 | 2.23 |
| Toluene ..... | 327 | 74 | 1.06 | 2.46 |
| Merthiolate (0.25%) | 307 | 73 | 1.00 | 2.43 |
| Anaerobic ($N_2$) ... | 287 | 67 | 0.93 | 2.23 |

(0.05 M $PO_4$ buffer pH 6.25; 1.5 mg spores/ml; 1% sugar.)

* $\mu$g/mg × hr—from slopes of linear curves; corrected for autolytic release of reducing sugars which was significant only where toluene added.

## Experimental Results

Metabolism of Sucrose: It can be reasoned that if sucrose were metabolized via invertase, then the invertase activity measured with suspensions of metabolizing spores should be less than that of dead spores or of spores whose assimilatory mechanisms are blocked by inhibitors. The magnitude of this difference should approximate the rate of metabolic utilization of the products of hydrolysis. Measurements summarizing certain phases of the metabolism of spores in solutions of sucrose in the presence and absence of sodium azide are presented in table I. The invertase activity of the suspension has not been affected by the azide, although respiration and assimilation have been almost completely inhibited. The sugar metabolized, as calculated from the increase in dry weight and from the respiration data, can be accounted for within the limits of experimental error by the decrease in sucrose remaining in the solution (unpublished). If sucrose were metabolized solely through the mediation of invertase, then blocking assimilation and respiration with azide could be expected to increase the production of reducing sugars by an amount equal to the rate of utilization of these

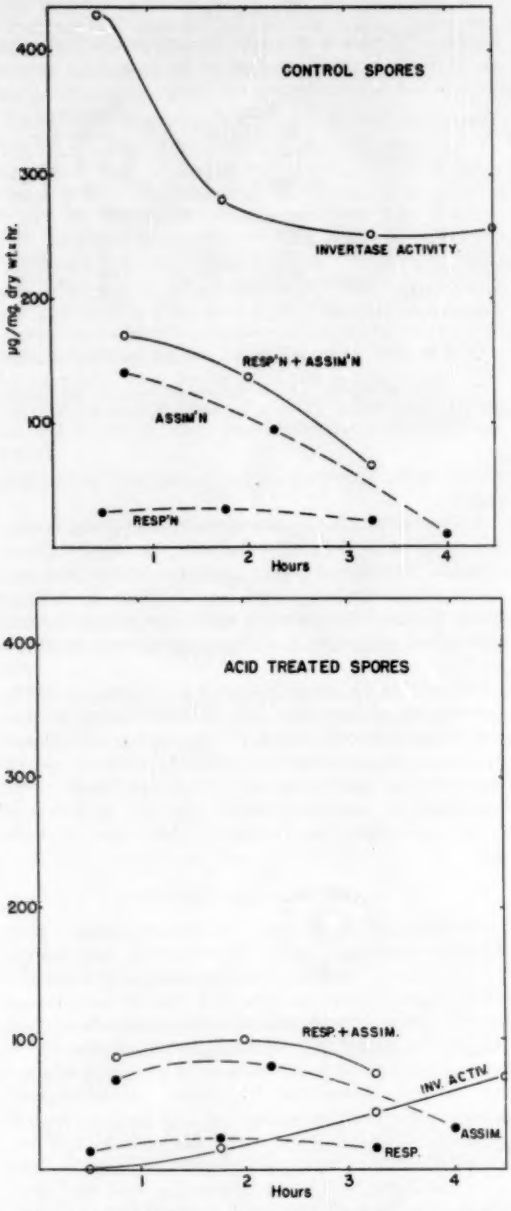


FIG. 1. Metabolism and hydrolysis of sucrose. A. Control spores. B. Acid treated spores. (Data in terms of μg sugar metabolized or hydrolyzed per mg dry wt of spores per hour.)

sugars—in other words, invertase activity in the presence of azide should be approximately 483 instead of 389. It can be inferred that sucrose enters the metabolism of the cell by some means other than through invertase action. Other methods of blocking metabolism—toluene, merthiolate, anaerobiosis—also fail to increase the rate of sucrose hydrolysis (table II). Heating to 60°C, a treatment which kills the spores but does not inactivate the invertase, does not increase the measured invertase activity.

A useful technique to develop further evidence on the role of invertase in sucrose metabolism would be a specific inhibitor for the enzyme. While no such agent is available, it is possible to completely inactivate the invertase of *M. verrucaria* spores by incubation with HCl at ca pH 1.0 without significantly affecting the viability of the spores or their rate of germination (10). Data showing the effect of acid treatment on various metabolic activities are presented in figure 1 A, B. No invertase activity in the treated spores is evident prior to one hour, but by two and one-half hours reducing sugars can be detected. This gradual increase in activity represents synthesis of invertase rather than reactivation (10). In spite of the extremely low invertase in the acid-treated spores, assimilation and oxidation of sucrose proceed at relatively rapid rates. The differences between the respiration and assimilation curves for control and acid-treated spores can be ascribed to acid injury, since other data show glucose metabolism as well as endogenous respiration to be similarly affected. In control spores the invertase activity is more than twice as great as to account for the combined respiration and assimilation. For the acid-treated spores, however, the utilization of sugar greatly exceeds the production of reducing sugars. In calculating these values it has been assumed that in the oxidation of sugar an R.Q. of 1.0 maintains, and also that all of the increase in dry weight is due to assimilation of sugar. Correction of this latter value to account for absorption of buffer components is very small and would be more than offset by the correction of the amount of sucrose oxidized calculated for an R.Q. of 1.2, which is closer to the actual value. The obvious conclusion is that sucrose can be metabolized at a rate approaching normal without the participation of invertase. In the experiment from which these data were taken the spores were washed to remove the acid. In other experiments the acid has been neutralized by adding buffer. Essentially the same results are obtained in both methods. In the experiment depicted in figure 1, invertase activity was measured without the addition of any poisons. It could be argued that the measured activity is not real, and that most of the reducing sugars formed are being metabolized. This point was checked in a similar experiment in which toluene was added periodically to a number of aliquots to follow the course of synthesis of invertase after acid treatment. Summarizing the data at two hours we find that metabolism of sucrose is twice the hydrolysis. Significant invertase activity cannot be demonstrated in acid-treated spores incubated with toluene, merthiolate, azide, ether or subjected to heat treatment (unpublished).

Further evidence relating to the path of metabolism of sucrose is found in a comparison of the rates of oxidation and assimilation of sucrose with that of its constituent sugars (fig. 2). The data lend strong

support to the concept of direct metabolism of sucrose since this sugar is respired and assimilated faster than its constituent sugars, single or combined.

One of the pathways by which sucrose can be metabolized, at least in certain organisms, is by phosphorolytic cleavage in which sucrose is split into glucose-1-phosphate and fructose by the enzyme sucrose phosphorylase in the presence of phosphate (4). This reaction is competitively inhibited by glucose (1). The action of several concentrations of glucose on the oxidation of sucrose and of fructose by spores is shown in table III. Low concentrations of glucose (1%) increase respiration on sucrose, while in the presence of higher concentrations the respiration is below that with sucrose alone. Fructose, however, does not suppress respiration on sucrose. In the presence of 10% glucose the respiration on sucrose is lowered to about that with glucose plus fructose. Without sucrose, respiration is more rapid as glucose

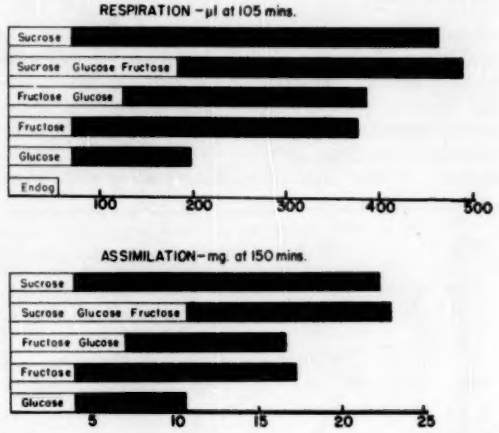


FIG. 2. Metabolism of sucrose and constituent sugars by spores. (Total concentration of sugar = 2% for assimilation; 1.33% for respiration.)

concentration is increased. High glucose increases somewhat the respiration on fructose.

Doudoroff *et al.* (2) have shown that sucrose phosphorylase is also capable of effecting arsenolysis of sucrose, the glucose-1-arsenate decomposing spontaneously into glucose and arsenate. The effect of arsenate on respiration with sucrose and related sugars is shown in table IV. Arsenate inhibits respiration on all of the substrates tested, but to varying degrees. It appears significant that the greatest inhibitory effect is found with sucrose, and furthermore that in the presence of arsenate, respiration on sucrose is equal to that on glucose + fructose. Endogenous respiration is stimulated slightly.

In view of the indications of some of the preceding data that a sucrose phosphorylase may be concerned in sucrose metabolism by these spores, several unsuccessful attempts to demonstrate the enzyme were made. The presence of both phosphatase and invertase in the spores interferes with the measurement of

TABLE III

EFFECT OF GLUCOSE AND FRUCTOSE ON RESPIRATION OF MYROTHECIUM VERRUCARIA SPORES IN PRESENCE OF SUCROSE

| SUBSTRATE | | | $Q_{O_2}$* | | RELATIVE $Q_{O_2}$* | |
|---|---|---|---|---|---|---|
| SUCROSE | GLUCOSE | FRUCTOSE | EXP. 1 | EXP. 2 | EXP. 1 | EXP. 2 |
| 1% | 0% | 0% | 16.9 | 16.5 | 1.00 | 1.00 |
| 1 | 1 | 0 | 18.1 | 18.1 | 1.07 | 1.09 |
| 1 | 10 | 0 | 14.5 | 15.0 | 0.86 | 0.91 |
| 1 | 25 | 0 | 14.1 | ... | 0.83 | ... |
| 1 | 0 | 10 | ... | 19.8 | ... | 1.2 |
| 0 | 0.5 | 0.5 | ... | 14.3 | ... | 0.87 |
| 0 | 10 | 0.5 | ... | 15.9 | ... | 0.96 |
| 0 | 1 | 0 | 11.5 | 11.2 | 0.68 | 0.68 |
| 0 | 10 | 0 | 12.3 | 14.4 | 0.73 | 0.87 |
| 0 | 25 | 0 | 13.9 | ... | 0.82 | ... |

(In buffer—0.05 M $PO_4$ at pH 6.25; Exp. 1—16 mg spores/vessel; Exp. 1—10 mg spores/vessel.)
* Not corrected for endogenous respiration.

phosphorylase. In experiments with whole spores, acid treatment was used to destroy the invertase. No phosphorylase activity could be demonstrated by measuring phosphate uptake of these spores in the presence of sucrose and phosphate when incubated under toluene. This may have been due to the fact that the phosphatase of the spores is not inactivated by acid treatment. Attempts to extract phosphorylase from ground spores following the procedure of Hassid *et al.* (4) in which fractional precipitation with $(NH_4)_2SO_4$ is employed were equally unsuccessful.

NATURE OF INVERTASE: Spores of *M. verrucaria* hydrolyze both sucrose and raffinose readily, yet do not hydrolyze melezitose, melibiose, nor turanose (tables V, VII and unpublished data). Glucose cannot be detected in the raffinose hydrolyzates. It is inferred that the enzyme in the spores is a fructosidase rather than a gluco-invertase (12). Several types of experiments were carried out to obtain data relating to the possibility that separate enzymes were involved in the hydrolysis of raffinose and sucrose by the spores. These experiments (unpublished) included: relative rates of hydrolysis of both substrates by spores and by spore extracts; determination of pH optimum for hydrolysis of both substrates; effect of

TABLE IV

EFFECT OF ARSENATE ON RESPIRATION

| SUBSTRATE | µL $O_2$ IN 60′ | | INHIBITION |
|---|---|---|---|
| | CONTROL | $+10^{-3}$ M ARSENATE | |
| Sucrose ..... | 326 | 189 | 42% |
| Glucose + fructose .. | 259 | 183 | 29 |
| Glucose ..... | 155 | 137 | 12 |
| Fructose ..... | 215 | 171 | 20 |
| Raffinose ..... | 118 | 103 | 13 |
| Endogenous ..... | 34 | 43 | −26 |

(12 mg spores/vessel; phthalate buffer pH 5.9; total sugar concentration—1%).

TABLE V

EFFECT OF ACID TREATMENT ON METABOLISM OF SUGARS BY MYROTHECIUM VERRUCARIA SPORES

| SUBSTRATE | TREATMENT | μG SUGAR PER MG SPORES PER HR | | | | |
|---|---|---|---|---|---|---|
| | | METABOLISM | | | HYDROLYSIS | |
| | | OXIDATION * | ASSIMILATION | OXIDATION + ASSIMILATION | − TOLUENE | + TOLUENE † |
| Sucrose | Control | 19 | 77 | 96 | 306 | 338 |
| | Acid treat. | 13 | 46 | 59 | 33 | 7.4 |
| Raffinose | Control | 9 | 33 | 42 | 129 | 204 |
| | Acid treat. | 4.8 | 7.8 | 13 | 8.6 | 6.3 |
| Melezitose | Control | 3.4 | 18 | 21 | 0 | 14 |
| | Acid treat. | 4.9 | 24 | 29 | 5.7 | 6.9 |
| Glucose | Control | 11 | 63 | 72 | .... | .... |
| | Acid treat. | 8 | 30 | 38 | .... | .... |

(Data are for 4 hours incubation with substrates.)
* Oxidation values calculated on basis of RQ = 1; not corrected for endogenous respiration.
† Values for hydrolysis under toluene are not corrected for autolysis.

raffinose and sucrose on resynthesis of the enzyme in acid-treated spores, activity being measured against both substrates. No evidence was encountered contradicting the assumption that a single enzyme is involved.

METABOLISM OF RAFFINOSE: Raffinose is respired and assimilated at rates approximately half those for sucrose and is hydrolyzed by metabolizing spores at about 40% of the rate for sucrose (table V). If metabolism is blocked or if killed spores are used, the apparent rate of raffinose hydrolysis is increased markedly. This increase is approximately equal to the rate of sugar utilization as calculated from the rates of respiration and assimilation (table II).

Acid treatment of spores renders them unable to hydrolyze raffinose and almost completely suppresses metabolism of this sugar (table V). Absorption of raffinose from the medium does not occur (table VI).

TABLE VI

METABOLISM OF SUGARS BY ACID-TREATED SPORES

| SUBSTRATE | RESPIRATION | TOTAL SUGAR IN SOLUTION | | |
|---|---|---|---|---|
| | | INITIAL | 1 HR | 3 HR |
| | *μl $O_2$ in 3 hr* | *mg/ml* | *mg/ml* | *mg/ml* |
| Raffinose ..... | 54 | 0.80 | 0.86 | 0.82 |
| Sucrose ..... | 216 | 1.12 | 0.76 | 0.0 |
| Glucose ..... | 124 | 1.09 | 0.90 | 0.38 |
| Endogenous ... | 42 | ... | ... | ... |

(5.5 mg acid-treated spores/ml; phosphate buffer pH 6.25.)

The metabolism of sucrose and raffinose differs also in the effect of arsenate on respiration with these sugars as substrates (table IV). Arsenate inhibits sucrose respiration markedly and raffinose respiration only slightly. Effects of glucose on raffinose respiration could not be tested with this organism, since glucose is respired more rapidly than is raffinose.

At this stage in the investigation the data were all consistent with the hypothesis that raffinose was metabolized hydrolytically via invertase. If this were valid, then only one-third of the raffinose molecule—the fructose portion—should be utilized. Melibiose should, therefore, accumulate in the medium since no melibiase has been detected in the spores (unpublished). Under conditions of normal growth more than one-third of the total sugar is utilized. Experiments designed to effect complete utilization of available sugar—i.e., dense spore suspensions and low concentrations of sugar—showed that melibiose is absorbed very slowly under these conditions (fig. 3). The data are thus not inconsistent with the original hypothesis.

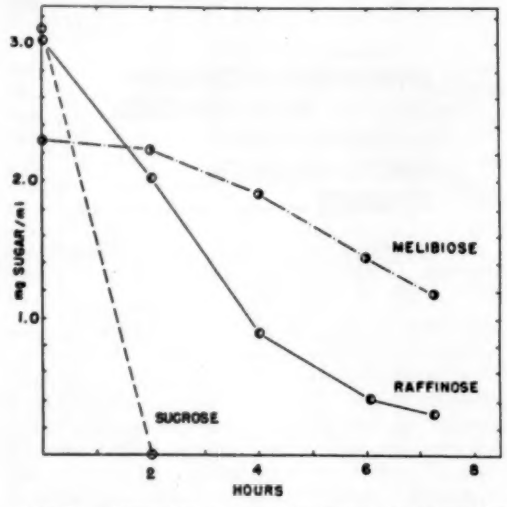


FIG. 3. Absorption of sugars by spores (ca 12 mg dry wt spores/ml; $PO_4$ buffer pH 6.25; data in terms of total sugars determined after acid hydrolysis).

METABOLISM OF MELEZITOSE: Melezitose is oxidized by *M. verrucaria* spores at rates significantly above

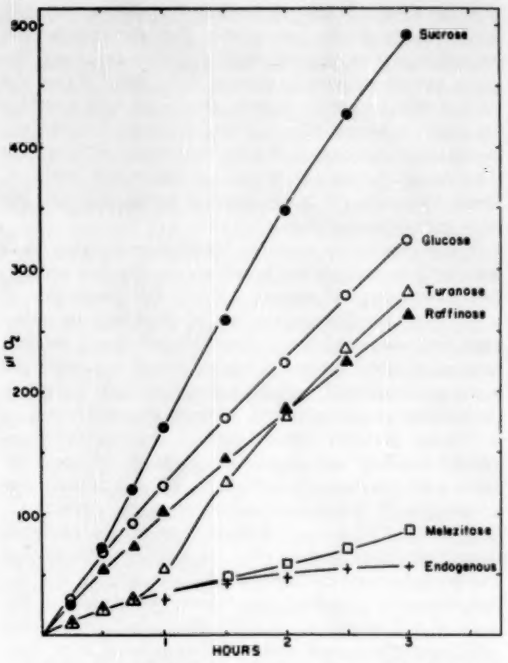


FIG. 4. Course of respiration of spores with various sugars. (5.1 mg spores/ml; 1% sugar; 0.05 M $PO_4$ buffer pH 6.25; 1.5 ml total volume in vessels.)

endogenous levels only after a lag of about an hour, the rate increasing gradually (fig. 4) and is assimilated slowly (table V). Measurable hydrolysis of this sugar does not occur, however. The small amount of reducing sugar noted in table IV where the spores were incubated under toluene with substrate is due to autolytic release of sugars. Acid treatment of the spores does not decrease metabolism of this sugar, but actually increases it slightly (table V).

TABLE VII

METABOLISM OF SUCROSE AND RELATED SUGARS

| SUBSTRATE | OXIDATION | ASSIMILATION | OXIDATION + ASSIMILATION | HYDROLYSIS |
|---|---|---|---|---|
| | $\mu g/mg \times hr$ | $\mu g/mg \times hr$ | | |
| Turanose .... | 17 | 94 | 111 | 0 |
| Melezitose .. | 4.7 | 10 | 15 | 0 |
| Sucrose ..... | 26 | 126 | 152 | .. |
| Endogenous .. | 2.4 | ... | ... | .. |

(Sugars at 1%; phosphate buffer pH 6.25; duration of experiment—2 hours; 4.35 mg spores/ml.)

METABOLISM OF TURANOSE: Turanose is respired and assimilated rapidly, although not as fast as sucrose (fig. 4, table VII), yet no hydrolysis can be detected by intact spores nor by spores in the presence of merthiolate or toluene. The shape of the respiration curve is suggestive of an adaptive mechanism. To determine whether an adaptive hydrolytic enzyme is formed, spores were incubated with turanose for three hours and were then tested for turanase activity after adding toluene to kill the cells. No hydrolytic activity was found. Arsenate inhibits respiration of turanose, yet with other disaccharides such as maltose (and also trehalose and cellobiose) stimulation occurs (fig. 5). This stimulation may be ascribed to effects upon endogenous respiration. Respiration on turanose is apparently suppressed by high concentrations of glucose—i.e., in the presence of turanose-glucose mixtures, respiration decreases at high glucose concentrations (table VIII). In this experiment the glucose or buffer was added from a second side arm at 100 minutes after adding the turanose, at which time the spores were respiring rapidly in linear fashion.

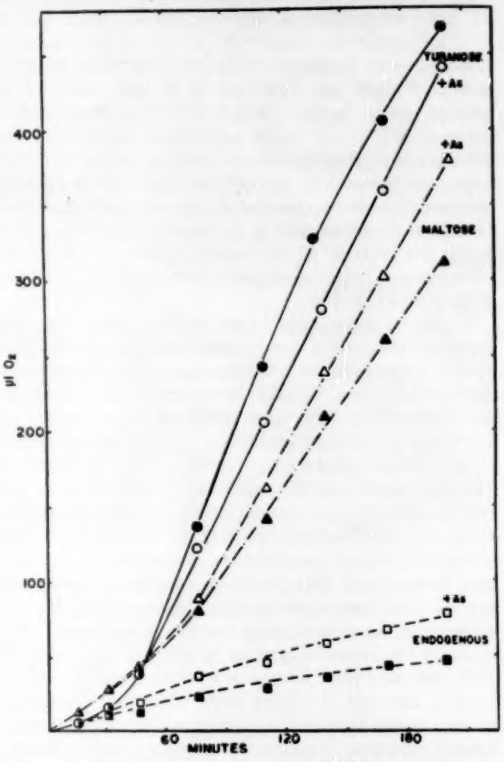


FIG. 5. Effect of arsenate on respiration of spores with turanose and maltose ($10^{-3}$ M arsenate; 0.05 M phthalate buffer pH 6; 8.3 mg spores/ml; 1% sugar; 1.5 ml total volume in vessels).

## DISCUSSION

Before proceeding with the interpretation of the data it should be pointed out that the spores of *M. verrucaria* do not germinate under the conditions and duration of experimentation employed in this study. The germination of these spores has been considered previously (8, 11).

TABLE VIII

EFFECT OF GLUCOSE ON TURANOSE RESPIRATION

| SUBSTRATE | | $Q_{O_2}$* | RELATIVE $Q_{O_2}$ |
|---|---|---|---|
| TURANOSE | GLUCOSE | | |
| 1% | 0% | 13.5 | 1.00 |
| 1 | 1 | 19.2 | 1.43 |
| 1 | 10 | 16.4 | 1.22 |
| 0 | 1 | 12.8 | 0.95 |
| 0 | 10 | 13.6 | 1.01 |

(10 mg spores/vessel; phosphate buffer pH 6.25.)
* Not corrected for endogenous respiration.

Metabolism of sucrose may be initiated by preliminary hydrolysis with invertase or by some non-hydrolytic pathway such as phosphorolysis, polymerization reaction (5, 6), or other unknown system/s. The presence of a great excess of invertase over the metabolic requirements of the cell, coupled with metabolic systems capable of utilizing glucose or fructose, would be expected to indicate a hydrolytic pathway. This is the situation in *M. verrucaria* spores, yet evidence from several types of experiments indicates this conclusion to be invalid.

If sucrose were metabolized via invertase, then the invertase activity of a suspension of cells as measured by the appearance of reducing sugars in the suspending medium should be a function of the metabolic activity of these cells, since the products of hydrolysis are being utilized in metabolism. Reduction of metabolism should produce a corresponding increase in reducing sugars in the medium, provided they are free to diffuse from the cells and that the conditions employed for blocking metabolism do not affect the actual activity of the enzyme. Experiments reported here have shown that blocking metabolism by a variety of techniques produces no increase in invertase activity of the spores when sucrose is substrate. Because of the reported surface location of the invertase (10), we need not be concerned with possible restrictions in the exit of sugars from the cells. In experiments with *Aspergillus luchuensis* spores (unpublished) blocking metabolism increases the measured invertase activity to the predicted magnitude. Results of additional studies are entirely compatible with a postulate of hydrolytic metabolism of sucrose via invertase with these spores.

Further evidence indicating non-hydrolytic metabolism of sucrose is found in comparing the rate of metabolism on sucrose with that on its products of hydrolysis. Both respiration and assimilation are greater on sucrose than on glucose-fructose mixtures.

Experiments using acid-treated spores substantiate the postulated nonhydrolytic metabolism. Willstätter and Lowry (15) have used this type of data to postulate direct fermentation of sucrose by yeast. Spores treated with 0.1 *N* HCl, or other acids, no longer display invertase activity, yet no comparable effects on cell viability nor on general metabolic activity are found. This is ascribed to the surface location of the enzyme (10). Study of sucrose metabolism in such acid treated spores has shown that respiration and assimilation are affected only slightly—to about the same extent as with a glucose substrate. Since the rate of metabolism on sucrose greatly exceeds hydrolysis, it is presumed that metabolism must proceed via some other pathway. While resynthesis of invertase does occur during the period of incubation with sucrose, this activity is inadequate to account for the high rate of metabolism.

These observations cannot be interpreted on a basis involving hydrolysis by invertase as the first step in the metabolism of sucrose. While the possibility of a pathway for the metabolism of disaccharides other than one mediated by hydrolytic carbohydrases had been recognized for many years, it was not until the work of Doudoroff and his colleagues that a definite mechanism was established through demonstration of a sucrose phosphorylase. Several reviews have appeared recently summarizing the status of our concepts and information regarding the significance and occurrence of direct metabolism of carbohydrates (2, 3, 4, 5, 6). In a critical evaluation of the evidence regarding direct fermentation of sucrose, maltose and lactose by yeast, Gottschalk (3) has refuted the direct schemes proposed by others primarily on the basis of pH relations, or lack of relation, of the cell with its environment and upon consideration of permeability of the cells to the sugars employed. He concludes that there is no evidence contraindicating a preliminary hydrolytic step. To explain the data presented here on a basis of preliminary hydrolysis requires several assumptions to be made to interpret each of the various lines of evidence. In brief, these assumptions would be: (1) postulation of an intracellular invertase whose activity cannot be demonstrated in control or acid-treated spores whose metabolic activity is blocked or destroyed by a variety of treatments; (2) this enzyme must be inhibited by glucose but not by fructose; (3) arsenate must block penetration of sucrose or inhibit the intracellular invertase; (4) following a brief lag in control spores, and a more extended one in acid treated spores (unpublished), sucrose must penetrate more rapidly than glucose and fructose. Space precludes a detailed analysis of the data on the basis of these assumptions which, in fact, do not permit completely satisfactory interpretations. We must infer that a non-hydrolytic pathway is much more probable. In seeking a mechanism for non-hydrolytic metabolism in *M. verrucaria* spores, the mediation of a sucrose phosphorylase appeared probable. While efforts to extract such an enzyme or to demonstrate phosphorolytic activity of intact cells—viable or under toluene—have been unsuccessful, certain indirect evidence is suggestive of the participation of this enzyme. The studies of Doudoroff *et al.* (1) have shown that sucrose phosphorylase is fundamentally a transglucosidase which is inhibited competitively by glucose. It would be anticipated, therefore, that glucose should suppress metabolism of sucrose by spores if sucrose phosphorylase is involved. Data presented verify this—high concentrations of

glucose suppress respiration to the same level as glucose-fructose mixtures. This would be the predicted behavior since blocking sucrose phosphorylase should shift the metabolism to invertase, the latter enzyme being present in more than adequate amounts. Other studies of Doudoroff (2) have shown that arsenate can be substituted for phosphate in the cleavage of sucrose by sucrose phosphorylase. In this case the glucose-arsenate formed hydrolyzes spontaneously, releasing free glucose. Thus if a comparable sucrose phosphorylase is involved in *M. verrucaria* spores, arsenate should suppress sucrose metabolism to the same level as with glucose-fructose mixtures, since only glucose and fructose are available, arising from either sucrose phosphorylase or from invertase activity. This prediction has been realized in measurements of the effects of arsenate on respiration on sucrose. Arsenate has no effect on the invertase activity of the spores.

Following this reasoning, it might be anticipated that metabolism of sucrose should be lowered to the level with glucose-fructose mixtures if phosphate is omitted from the medium. Experiments have shown, however, that sucrose metabolism is not affected by the presence or absence of phosphate. Since 1.4% of the dry weight of the spores is phosphorus, it must be presumed that this is adequate for the postulated mechanism. Indeed, the reserves of all inorganic nutrients are adequate to satisfy the metabolic requirements for at least five or six hours since assimilation and respiration on sugars are not greatly influenced by omitting nitrogen, phosphate, magnesium, sulfate or potassium.

We must now explain the more rapid rate of metabolism on sucrose than on a mixture of glucose and fructose. Assuming that a phosphorolytic mechanism is involved, both glucose-1-phosphate and fructose are present within the cell or are available to it if the enzyme is at the cell surface. Since invertase is also present, glucose is also available. Thus cells suspended in sucrose are presumably metabolizing all three of these sugars. The more rapid metabolism on sucrose than on glucose plus fructose is thus ascribed, hypothetically, to the additional metabolite glucose-1-$PO_4$. It must be assumed further that the glucose-1-$PO_4$ is formed within the cell since attempts to duplicate this effect by adding glucose-1-phosphate extracellularly were unsuccessful (unpublished). Presumably permeability of the cell to the phosphorylated sugar is limiting, since the endogenous respiration is increased only very slightly when this compound is added.

The trisaccharides, raffinose and melezitose, both contain sucrose (fig. 6). In raffinose a galactose residue is attached to the glucose portion of the sucrose moiety, the galactose-glucose linkage being the same as that in melibiose. Raffinose can thus be considered as a combination of sucrose and melibiose, with the glucose being common to both disaccharides. In melezitose an extra glucose is linked to the fructose portion of the sucrose in the same manner as in the disaccharide, turanose. Melezitose can thus be considered as a combination of sucrose and turanose, with the fructose group being common to both sugars. The composition of these oligosaccharides and the enzymes responsible for their hydrolysis are indicated in figure 6. Data presented here indicate that of the four enzymes which may be concerned in the hydrolysis of these sugars, only fructo-invertase is present in *M. verrucaria* spores in measurable quantities. In spite of the absence of enzymes which can hydrolyze melezitose or turanose, both these sugars are metabolized —the former slowly, the latter quite rapidly. Such data are strongly indicative of non-hydrolytic metabolism of these two sugars. It is interesting to note in this connection that the rate of hydrolysis of raffinose by spores is increased if metabolism is blocked, and that the magnitude of increase is about equal to the rate of utilization in metabolism. These observations are all consistent with the postulated non-hydrolytic metabolism of sucrose. It is possible that the same enzyme—a transglucosidase—may be responsible for the metabolism of sucrose, turanose and melezitose, all of which have an exposed $\alpha$-glucopyranose group in contrast to the internal location of this group is raffinose. It must be emphasized that while circumstantial evidence points toward a sucrose phosphorylase, failure to detect this enzyme may be suggestive of some other pathway.

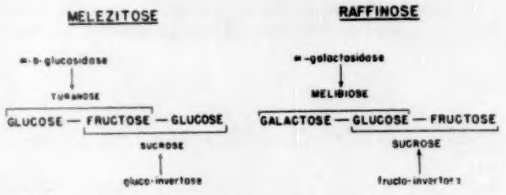


FIG. 6. Structures and enzymic mechanisms of hydrolysis of the non-reducing trisaccharides, raffinose and melezitose (after Pigman and Goepp, 1948).

## SUMMARY

(1) Data from four types of experiments indicate that sucrose is metabolized by a non-hydrolytic system in spores of the fungus *Myrothecium verrucaria* even though invertase is present in excess of the metabolic requirements. The evidence available is consistent with the postulate that sucrose metabolism is mediated by a sucrose phosphorylase, although attempts to demonstrate such an enzyme were unsuccessful.

(2) Melezitose and turanose are not hydrolyzed by the spores and thus appear to be metabolized by a non-hydrolytic system.

(3) Evidence indicates that raffinose is metabolized via invertase, the enzyme being a fructosidase.

The author is indebted to Miss Anna B. Norton for her technical assistance, to Dr. E. T. Reese for the glucose oxidase, and to Mr. H. S. Levinson and Dr. E. T. Reese for their critical review of the manuscript.

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# PEANUT AND OIL PALM FOLIAR DIAGNOSIS INTERRELATIONS OF N, P, K, Ca, Mg[1]


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Foliar diagnosis is now extensively used as a research tool to evaluate the nutritional status of plants. Its definition and name, "diagnostic foliaire," was first used in France by Lagatu and Maume (8) in their pioneer work, although many other workers had already analyzed plant materials to determine the nutritional requirements of cultivated plants.

In Sweden, Lundegårdh (9) established some fundamental concepts regarding the techniques of plant analysis in his extensive researches on oats and in the United States the important work of Thomas (19) attracted attention to foliar diagnosis. A comprehensive historical review of the subject has been given by Goodall and Gregory (7) and more recently by Ulrich (10).

Since the French literature on this subject is not readily available in English-speaking countries it may be well to mention recent work done in France. Maume *et al.* worked on grape vines (11, 12, 13) and on corn (14); Bouat, Renaud, and Dulac worked on olive trees (1); Franc de Ferriere worked on grape vines (5), corn, and potatoes (6). Also, many papers can be found in *C. R. Académie des Sciences* and *C. R. Académie d'Agriculture.*

This paper deals with the results of foliar diagnoses obtained with the peanut (*Arachis Hypogaea* L.) and the oil palm (*Elaeis Guineensis* Jacq.) in French Africa. The work reported for the peanut plant is based on the physiological researches of Burkhardt and Page (3) and of Prevot (15), who laid down the principles of leaf sampling for this plant in Africa (16), and on the many field experiments conducted in Africa during 1950 and 1951 (17, 18). For the oil palm we started with the basic work of Chapman and Gray (4), whose techniques we applied at our research stations in 1951. Lack of space prevents us from giving complete results of the experiments, but we shall briefly describe a typical experiment and summarize the other results.

## Methods

The experiments were conducted at twelve different locations in French Africa (fig. 1), with climatic and ecological conditions ranging from those of Sénégal to those of French Moyen-Congo, which provided a good basis for a generalization of some of the conclusions.

The leaf sampling techniques for the peanut are those described by Prevot and Ollagnier (17) and for

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the oil palm by Chapman and Gray (4). The first leaf of the main stem of the peanut is taken off at the beginning of flowering, while for the oil palm the first completely expanded but not senescent leaf is chosen. Ten median leaflets of the oil palm leaf, from which are removed the midrib, both ends, and marginal areas, leaving only the middle part of each leaflet, are used for analysis. A medium-sized sample contains the leaves of fifty peanut plants or of twenty-five oil palm trees.

an appreciable factor in thousands of analyses, although this would have been a good method otherwise. A milliequivalent basis, while useful in the comparison of K, Ca, and Mg, is hardly of value when polyvalent ions come into consideration.

Our present conclusions (1952) are based on the results of nine experiments on the peanut and six on the oil palm. For the peanut the experimental designs were of the complex factorial type $3^3$ (the three elements N, P, K at three levels 0, 1, 2) or $2^3$ (the three

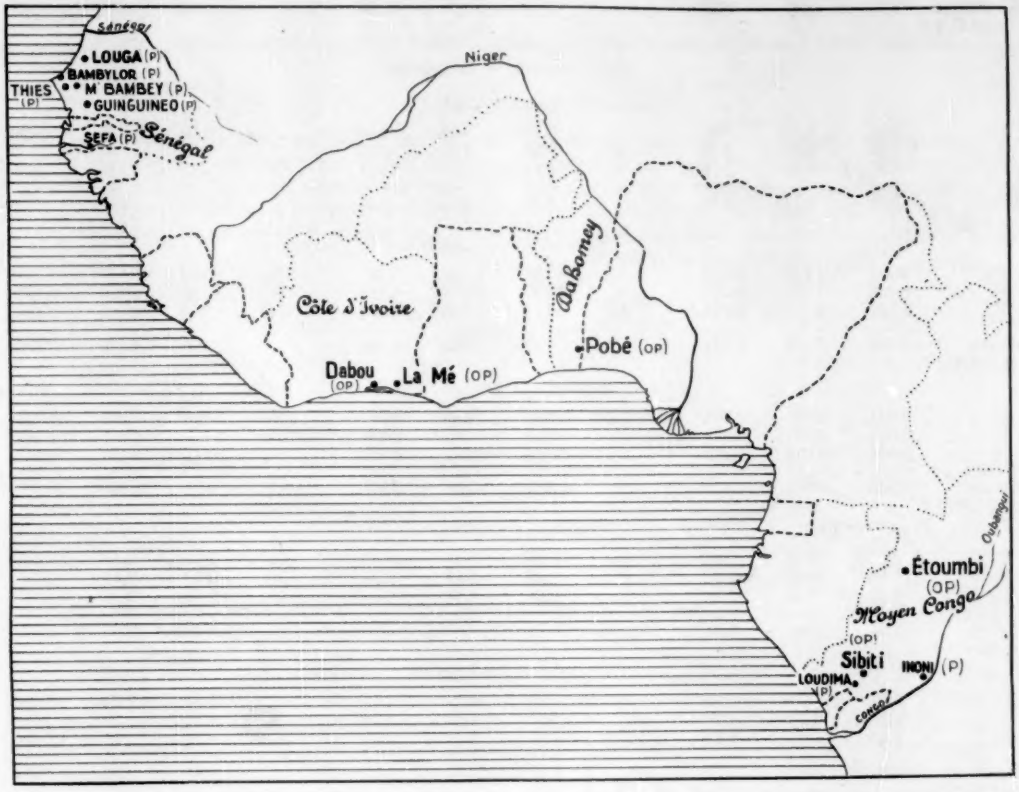



FIG. 1. Location of different experimental fields in Africa. P = Peanut; OP = Oil Palm.

Consistency of procedure is of prime importance in leaf sampling; tissue samples and sampling times must be comparable for valid results.

The samples are dried in Africa and then air mailed to Paris. Comparative chemical analysis in Africa and Paris have shown no important modifications in the samples. The chemical analysis done in Paris are N (semimicro Kjeldahl), P (Lorenz Scheffer), K, Ca, and Mg (Beckman flame spectrophotometer).

The results of the chemical analysis are expressed as elements (N, P, K, Ca, and Mg) on a percentage-dry-weight basis, rather than as oxides as usually done in agronomical papers. Nor did we express the results in milligram atoms as done by Lundegårdh (9) and Prevot (15) since this complicates the calculations,

elements N, P, K at two levels 0, 1), generally with partial confounding. With the oil palm we had to use experiments already started, and these were of the randomized design.

## Experimental Results on Peanut

Results: A typical peanut experiment (18) was performed in 1951 at the Centre de Recherches Agronomiques de M'Bambey (see fig. 1) by MM. Gillier and Orgias, Research Assistants of the Institut de Recherches pour les Huiles et Oléagineux. This was a $3 \times 3 \times 3$ factorial experiment with partial confounding; i.e., eight-one plots arranged in nine subblocks of nine plots each. A part of the second order interaction is confounded with block differences. The

TABLE I

RESULTS OF A PEANUT EXPERIMENT
CONTINGENCY TABLES

| | $N_0$ | $N_1$ | $N_2$ | | $P_0$ | $P_1$ | $P_2$ | | $K_0$ | $K_1$ | $K_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *Yields in kg per ha* (± *58.1*) † | | | | | | | |
| $P_0$ | 1936 | 2201 | 2220 | $K_0$ | 2011 | 2472 | 2416 | $N_0$ | 2140 | 2303 | 2302 |
| $P_1$ | 2449 | 2634 | 2440 | $K_1$ | 2255 | 2557 | 2689 | $N_1$ | 2401 | 2676 | 2474 |
| $P_2$ | 2377 | 2716 | 2647 | $K_2$ | 2091 | 2494 | 2635 | $N_2$ | 2342 | 2520 | 2445 |
| Means (± 33.5) ‡ | 2254 | 2517 | 2436 | | 2119 | 2507 | 2580 | | 2300 | 2500 | 2407 |
| | | | | *Leaf elements in % dry wt* | | | | | | | |
| | | | | *Nitrogen* (± *0.058*) † | | | | | | | |
| $P_0$ | 3.67 | 3.82 | 3.78 | $K_0$ | 3.76 | 3.69 | 3.63 | $N_0$ | 3.70 | 3.68 | 3.68 |
| $P_1$ | 3.72 | 3.73 | 3.81 | $K_1$ | 3.80 | 3.81 | 3.71 | $N_1$ | 3.62 | 3.81 | 3.75 |
| $P_2$ | 3.67 | 3.63 | 3.76 | $K_2$ | 3.71 | 3.76 | 3.71 | $N_2$ | 3.75 | 3.84 | 3.76 |
| Means (± 0.034) ‡ | 3.69 | 3.72 | 3.78 | | 3.76 | 3.75 | 3.69 | | 3.69 | 3.78 | 3.73 |
| | | | | *Phosphorus* (± *0.0078*) † | | | | | | | |
| $P_0$ | 0.137 | 0.143 | 0.138 | $K_0$ | 0.141 | 0.165 | 0.161 | $N_0$ | 0.155 | 0.153 | 0.153 |
| $P_1$ | 0.156 | 0.166 | 0.172 | $K_1$ | 0.137 | 0.171 | 0.169 | $N_1$ | 0.152 | 0.164 | 0.154 |
| $P_2$ | 0.169 | 0.162 | 0.173 | $K_2$ | 0.141 | 0.156 | 0.173 | $N_2$ | 0.160 | 0.160 | 0.163 |
| Means (± 0.0045) ‡ | 0.154 | 0.157 | 0.161 | | 0.140 | 0.164 | 0.168 | | 0.156 | 0.159 | 0.157 |
| | | | | *Potassium* (± *0.055*) † | | | | | | | |
| $P_0$ | 0.861 | 0.859 | 0.864 | $K_0$ | 0.837 | 0.826 | 0.913 | $N_0$ | 0.899 | 0.889 | 0.992 |
| $P_1$ | 0.925 | 0.840 | 0.877 | $K_1$ | 0.805 | 0.837 | 0.894 | $N_1$ | 0.835 | 0.817 | 0.883 |
| $P_2$ | 0.904 | 0.836 | 0.871 | $K_2$ | 0.942 | 0.980 | 0.893 | $N_2$ | 0.841 | 0.831 | 0.939 |
| Means (± 0.0318) ‡ | 0.927 | 0.845 | 0.871 | | 0.861 | 0.881 | 0.900 | | 0.858 | 0.846 | 0.938 |
| | | | | *Calcium* (± *0.072*) † | | | | | | | |
| $P_0$ | 2.15 | 2.33 | 2.37 | $K_0$ | 2.22 | 2.43 | 2.32 | $N_0$ | 2.19 | 2.19 | 2.30 |
| $P_1$ | 2.32 | 2.52 | 2.51 | $K_1$ | 2.29 | 2.52 | 2.34 | $N_1$ | 2.42 | 2.44 | 2.34 |
| $P_2$ | 2.19 | 2.36 | 2.44 | $K_2$ | 2.34 | 2.40 | 2.32 | $N_2$ | 2.37 | 2.52 | 2.42 |
| Means (± 0.041) ‡ | 2.22 | 2.40 | 2.44 | | 2.28 | 2.45 | 2.33 | | 2.32 | 2.38 | 2.35 |
| | | | | *Magnesium* (± *0.059*) † | | | | | | | |
| $P_0$ | 1.16 | 1.23 | 1.26 | $K_0$ | 1.17 | 1.22 | 1.23 | $N_0$ | 1.14 | 1.23 | 1.20 |
| $P_1$ | 1.21 | 1.33 | 1.28 | $K_1$ | 1.27 | 1.39 | 1.28 | $N_1$ | 1.22 | 1.36 | 1.20 |
| $P_2$ | 1.19 | 1.21 | 1.33 | $K_2$ | 1.22 | 1.21 | 1.23 | $N_2$ | 1.25 | 1.35 | 1.26 |
| Means (± 0.034) ‡ | 1.19 | 1.26 | 1.29 | | 1.22 | 1.27 | 1.25 | | 1.20 | 1.31 | 1.22 |

Standard error for individual results: †.
Standard error for means: ‡.

treatments, all combinations of the three elements N, P, and K at three levels, are as follows:

| | | | | | | |
|---|---|---|---|---|---|---|
| $N_0$ | 0 | $P_0$ | 0 | $K_0$ | 0 | |
| $N_1$ | 37.5 kg/ha | $P_1$ | 75 kg/ha | $K_1$ | 37.5 | kg/ha |
| $N_2$ | 75 kg/ha | $P_2$ | 150 kg/ha | $K_2$ | 75 | kg/ha |

N applied as ammonium sulfate;
P applied as dicalcium phosphate;
K applied as potassium chloride.
ha = hectare.

All applications were made as side-dressing five centimeters from the row and five centimeters deep. The variety used was the 24-11 strain of M'Bambey, a runner type. The results are given in tables I, II, and III.

DISCUSSION: For abbreviation we shall indicate the treatments by $N_T$, $P_T$, and $K_T$, and the leaf contents by $N_L$, $P_L$, $K_L$, $Ca_L$, and $Mg_L$.

The yields (table I) show a predominating influence of $P_T$, a lesser effect of $N_T$, and a small effect of $K_T$, being just within the 0.05 significant level. However, when there is a highly significant correlation between yield and $P_L$ (0.01 level of significance, table III), there is no such correlation between yield and $N_L$ or $K_L$. There is even a tendency for a negative correlation between yield and $K_L$. These relationships are

TABLE II

LINEAR AND CURVATURE EFFECTS OF THE FERTILIZER TREATMENTS ($N_T$, $P_T$, $K_T$) ON LEAF CONTENTS ($N_L$, $P_L$, $K_L$, $Ca_L$, $Mg_L$) AND ON YIELD

| | LINEAR EFFECT | | | | | | CURVATURE EFFECT | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | YIELD | $N_L$ | $P_L$ | $K_L$ | $Ca_L$ | $Mg_L$ | YIELD | $N_L$ | $P_L$ | $K_L$ | $Ca_L$ | $Mg_L$ |
| $N_T$ | +182 ** | +0.10 * | +0.007 | −0.056 | +0.21 ** | +0.10 * | −345 ** | +0.02 | +0.002 | +0.107 | −0.14 | −0.04 |
| $P_T$ | +461 ** | −0.07 | +0.028 ** | +0.039 | +0.04 | +0.03 | −316 ** | −0.06 | −0.021 * | −0.000 | −0.30 ** | −0.08 |
| $K_T$ | +107 * | +0.04 | +0.001 | +0.080 | +0.03 | +0.01 | −294 ** | −0.13 | −0.005 | +0.106 | −0.09 | −0.20 * |
| Standard error | ± 47.4 | ± 0.048 | ± 0.0063 | ± 0.0451 | ± 0.059 | ± 0.048 | ± 82.2 | ± 0.083 | ± 0.0109 | ± 0.0781 | ± 0.101 | ± 0.083 |

Linear effect of $N = N_2 - N_0$.
Curvature effect of $N = N_2 - 2N_1 + N_0$.

* Significant at 5%.
** Significant at 1%.

shown in figure 2, where the main effects are plotted against yields and leaf contents. The results indicate that P is the principal deficient element and that, on the basis of this experiment, a fertilizer formula with a predominating proportion of P should be recommended.

positive correlation $P_L$:$K_L$ not as a correlation of absorption capacity but rather as an improvement in the physiological condition of the plant, due to the favorable action of the most deficient element, P. These more favorable physiological conditions would tend to increase accumulation ability.

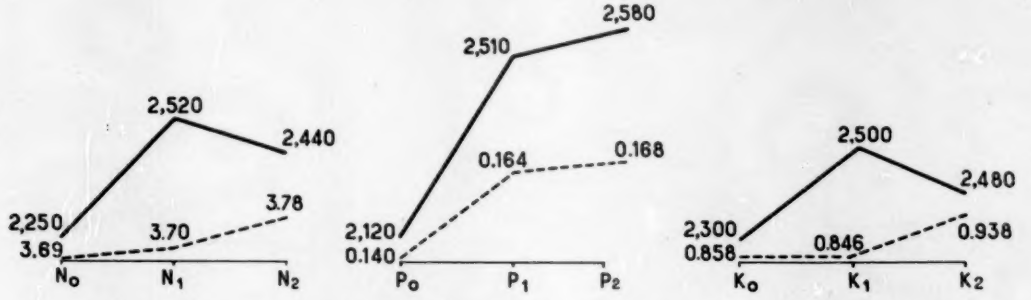



FIG. 2. Main effects of N, P or K on yields (unshelled nuts kg/ha) and leaf contents (gm % dry weight) of N, P or K. 1 cm represents a signicant difference at 5% between main effects.

The favorable effects of $K_T$ and $N_T$ upon yields may act indirectly. For example, the favorable effect of $K_T$ might be associated with the uptake of P, as there is a significant (0.05) positive correlation between $K_L$ and $P_L$ (table III). However, this does not seem to be the case, for it can be seen that $K_{T2}$ tends to act the opposite way in the presence of $P_{T1}$. For instance, we see (table I) that $K_{T2}$ increases $K_L$ from 0.837 to 0.980 (significant) and decreases $P_L$ from 0.171 to 0.156 (significant). We interpret the

The correlation between $N_L$ and $P_L$, although positive, is not significant (table III). This is related to the strong negative correlation between $N_L$ and $K_L$ along with the positive correlation between $K_L$ and $P_L$.

It is possible that the favorable effect of $N_T$ is partially related to the increase in $Ca_L$ and $Mg_L$ due to this treatment (tables I and III). As a matter of fact, we find a highly significant correlation between yield and $Ca_L$ or $Mg_L$, which would seem to indicate a favorable effect of Ca and Mg, a point to be investigated later.

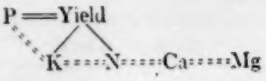
TABLE III

CORRELATIONS

| BETWEEN | YIELD | $P_L$ | $K_L$ | $Ca_L$ | $Mg_L$ |
|---|---|---|---|---|---|
| $N_L$ | +0.1233 | +0.0690 | −0.4464 ** | +0.5763 ** | +0.5797 ** |
| $P_L$ | +0.8096 ** | ..... | +0.2658 * | +0.0760 | −0.0347 |
| $K_L$ | −0.1880 | ..... | ..... | −0.6521 ** | −0.7575 ** |
| $Ca_L$ | +0.6641 ** | ..... | ..... | ..... | +0.8713 ** |
| $Mg_L$ | +0.5390 ** | ..... | ..... | ..... | ..... |

* Significant at 5%.
** Significant at 1%.

The main results of this experiment can be summarized as follows:




The solid lines show relations between yield and treatments and dotted lines correlations between elements.

## General Survey of Experiments

Results: Lack of space prevents us from presenting complete results of each analysis; however, we have summarized them in table IV (peanut) and table V (oil palm) by giving only the results with and without the favorable treatment. Also, for comparison, we have included some data of Chapman and Gray (4), recalculated on a basis of percentage of dry weight.

Discussion: It should be pointed out that all favorable treatments of both the peanut and the oil palm increased the leaf content of the same element, except at Inoni and Louga. The Inoni experiment gave no significant results, and the Louga exception, discussed in another paper (18), is related to the very low level of N in the soils which had not been corrected by fertilizer application (50 kg of ammonium sulfate per hectare). This would be a case of being in the "minimum percentage" range, according to Macy (10).

So, the treatment that increases yield also corrects deficiency of the element in the leaf. Once more the validity of foliar diagnosis for finding mineral deficiencies in soils is confirmed. In a recent experiment with the peanut Bouyer (2) failed to find such a relation; however, both his leaf sampling and experimental design were quite different from ours.

*Critical levels:* We give to the critical level a practical goal and *define it as the concentration of the element in the leaf (dry weight basis) above which a yield response from the element in the fertilizer is unlikely to occur.*

This concept is of a major importance for practical use, as rightly pointed out by Ulrich (20), because it allows advising manures which will give the most economic returns.

Critical levels are indicated by $N_c$ for $N_L$, $P_c$ for $P_L$, and $K_c$ for $K_L$. To determine them we use either a direct or an indirect method.

Table IV

General Peanut Results—Leaf Analysis

| Location | Number of replicates | Treatment | N | P | K | Ca | Mg | N/P | K+Ca+Mg | Yields | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % of dry weight | | | | | | | Kg/ha | % |
| *Sénégal* | | | | | | | | | | | |
| Thiès 1950 | 27 | (–) | 4.46 | 0.483 | 0.534 | 0.83 | 0.86 | 9.2 | 2.22 | 574 | 100 |
| | | K | 4.31 | 0.395 | 0.652 | 0.75 | 0.71 | 10.9 | 2.11 | 819 | 143 |
| Séfa 1950 | 27 | (–) | 4.65 | 0.165 | 1.61 | 1.16 | 0.489 | 28.2 | 3.26 | 1580 | 100 |
| | | P | 4.75 | 0.194 | 1.49 | 1.35 | 0.605 | 24.5 | 3.44 | 2040 | 129 |
| Séfa 1951 | 27 | (–) | 4.58 | 0.179 | 2.14 | 1.79 | 0.725 | 25.6 | 4.65 | 1654 | 100 |
| | | P | 4.84 | 0.256 | 1.99 | 1.93 | 0.847 | 18.9 | 4.73 | 2082 | 126 |
| Bambey 1951 | 27 | (–) | 3.76 | 0.140 | 0.861 | 2.28 | 1.22 | 26.9 | 4.36 | 2119 | 100 |
| | | P | 3.69 | 0.168 | 0.900 | 2.33 | 1.25 | 22.9 | 4.60 | 2500 | 122 |
| Bambylor 1951 | 27 | (–) | 3.42 | 0.153 | 0.776 | 1.96 | 1.13 | 22.3 | 3.87 | 1801 | 100 |
| | | P | 3.34 | 0.176 | 0.774 | 2.06 | 1.23 | 19.0 | 4.03 | 2248 | 125 |
| Louga 1951 | 20 | (–) | 2.91 | 0.168 | 1.66 | 1.24 | 0.403 | 17.3 | 3.30 | 976 | 100 |
| | | N | 2.76 | 0.156 | 1.63 | 1.33 | 0.410 | 17.7 | 3.37 | 1210 | 124 |
| Guinguinéo 1951 | 20 | (–) | 4.36 | 0.240 | 1.18 | 2.23 | 1.06 | 18.2 | 4.47 | 1556 | 100 |
| | | K | 4.31 | 0.235 | 1.24 | 2.27 | 1.08 | 18.3 | 4.59 | 1840 | 118 |
| *French Congo* | | | | | | | | | | | |
| Loudima 1951 | 27 | (–) | 3.21 | 0.139 | 2.25 | 2.40 | 0.305 | 23.1 | 4.95 | 2227 | 100 |
| | | Mg | 3.17 | 0.142 | 2.23 | 2.42 | 0.344 | 22.3 | 4.99 | 2324 | 104 |
| Inoni 1951 | 18 | (–) | 3.48 | 0.228 | 1.90 | 1.18 | 0.409 | 15.3 | 3.49 | 1139 | 100 |
| | | Ca | 3.70 | 0.209 | 1.82 | 1.16 | 0.555 | 17.7 | 3.53 | 1209 | 106 |

The *direct method* consists of comparing yield response to leaf analysis.

For $N_c$ of the peanut, for instance, we found the following values for $N_L$ in the plots without $N_T$ and for the yield response to the application of nitrogen:

| | $N_L$ | Yield response to the application of nitrogen |
|---|---|---|
| Louga (1951) | 2.91 | +24% ** |
| Bambylor (1951) | 3.36 | +16% ** |
| Bambey (1951) | 3.69 | +12% ** |
| Guinguinéo (1951) | 4.27 | 0 |
| Thiès (1950) | 4.42 | + 5% |
| Séfa (1950) | 4.60 | + 2% |
| Séfa (1951) | 4.70 | + 1% |

We see that the yield responses decrease as $N_L$ increases from 2.91 to 4.27.

TABLE V

GENERAL OIL PALM RESULTS—LEAF ANALYSIS

| LOCATION | AGE | TREATMENT | NUMBER OF REPLICATES | N | P | K | Ca | Mg | N/P | K+Ca+Mg | YIELDS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | % OF DRY WEIGHT | | | | | | | KG/HA | % |
| POBÉ | | | | | | | | | | | | |
| (Dahomey) | Nursery | .. | 5 | 3.63 | 0.220 | 1.80 | 0.650 | 0.470 | 16.5 | 2.92 | No production | |
| | 2-year-old trees | (–) | 5 | 2.78 | 0.168 | 0.697 | 0.700 | 0.540 | 16.5 | 1.94 | No production | |
| | | K | | 2.75 | 0.171 | 1.08 | 0.721 | 0.432 | 16.1 | 2.23 | | |
| | 20-year-old trees | (–) | 6 | 2.54 | 0.167 | 0.499 | 0.932 | 0.651 | 15.2 | 2.08 | 4730 | 100 |
| | | K | | 2.64 | 0.171 | 0.872 | 0.776 | 0.470 | 15.4 | 2.12 | 6830 | 147 |
| DABOU | | | | | | | | | | | | |
| (Ivory Coast) | 20-year-old trees | (–) | 5 | 3.01 | 0.193 | 0.496 | 0.589 | 0.318 | 15.6 | 1.40 | 3100 | 100 |
| | | K | | 2.83 | 0.180 | 0.765 | 0.756 | 0.286 | 15.7 | 1.81 | 9350 | 302 |
| SIBITI | | | | | | | | | | | | |
| (French Congo) | 7-year-old trees | Healthy | 11 | 2.80 | 0.162 | 1.38 | 0.457 | 0.230 | 17.3 | 2.07 | No records | |
| | | Unhealthy | | 2.53 | 0.158 | 1.54 | 0.412 | 0.202 | 16.0 | 2.15 | No records | |
| ETOUMBI | | | | | | | | | | | | |
| (Moyen Congo) | 20-year-old trees | Control | 6 | 2.81 | 0.184 | 0.91 | 0.530 | 0.118 | 15.3 | 1.56 | 3920 | 100 |
| | | NPK | | 2.95 | 0.189 | 1.21 | 0.562 | 0.114 | 15.6 | 1.89 | 4210 | 107 |
| | | NPK+Mg | | 2.98 | 0.197 | 1.13 | 0.577 | 0.135 | 15.1 | 1.84 | 5520 | 141 |
| MALAYA (from Chapman) | | | | | | | | | | | | |
| Experiment 1 | | (–) | .. | 2.26 | 0.128 | 1.01 | .... | .... | 17.6 | ... | Not pub. | 100 |
| | | P | | 2.45 | 0.153 | 0.96 | .... | .... | 16.0 | ... | Not pub. | 193 |
| Experiment 2 | | (–) | .. | 2.58 | 0.239 | 0.560 | 0.960 | 0.376 | 10.8 | 1.90 | Not pub. | 100 |
| | | K | | 2.73 | 0.247 | 1.09 | 0.832 | 0.329 | 11.1 | 2.25 | Not pub. | 168 |

The $N_c$ has been approximated to $N_c = 4.00$. The same reasoning applies to other critical-level values.

The *indirect method*, or examination of special cases, may also give some information. For instance, at Dabou $K_T$ (which increases yield 200%) lowers $N_L$ from 3.01 to 2.83. In Malaya (Chapman) this same $K_T$ with a yield increase of 90% brings $N_L$ from 2.58 up to 2.73. Thus, the $N_c$ may be situated around 2.70. Similar reasoning may be used in other cases.

Summarizing, we propose the following critical levels:

| Peanut | Oil palm |
|---|---|
| $N_c = 4.00$ | $N_c = 2.70$ |
| $P_c = 0.20$ | $P_c = 0.15$ |
| $K_c = 1.20$ | $K_c = 1.00$ |

These critical levels should be considered approximate; they will probably have to be modified in the course of further experiments involving varietal and climatic influences. However, it is interesting to point out the similarity of the $K_c$ of the oil palm in Chapman's experiments in Malaya and our experiments in different African regions.

*Interrelations of elements:* While the critical levels are of major importance they do not, of course, tell us the whole story. In order to understand some experimental results more fully it is necessary to consider the interrelationships of the different elements.

*Ratios between elements:* The $N_L/P_L$ ratio is surprisingly constant. For the oil palm (table V) it is about the same in seedlings, young or old trees (Pobé); also, it is about the same in such different conditions as Malaya and Africa. Its value is about 16 and, in a general way, a favorable treatment brings it down or up when it is lower or higher than 16 in the control.

This ratio is less constant in the peanut (table IV); however, it tends toward a certain value under a

TABLE VI

PEANUT—RATIOS BETWEEN N AND P

| LOCATION | TREATMENT | N:P | TREND OF N:P | YIELD INCREASE |
|---|---|---|---|---|
| Thiès | (–) | 9.2 | ↓ | +43% |
| | K | 10.9 | | |
| Inoni | (–) | 15.3 | ↓ | + 6% |
| | Ca | 17.7 | | |
| Louga | (–) | 17.3 | ↓ | +24% |
| | N | 17.7 | | |
| Guinguinéo | (–) | 18.2 | ↓ | +18% |
| | K | 18.3 | | |
| | | | N:P = 20 | |
| Bambylor | P | 19.0 | ↑ | +25% |
| | (–) | 22.3 | | |
| Loudima | Mg | 22.3 | ↑ | + 4% |
| | (–) | 22.9 | | |
| Séfa 51 | P | 18.9 | ↑ | +26% |
| | (–) | 25.6 | | |
| M'Bambey | P | 22.9 | ↑ | +22% |
| | (–) | 26.9 | | |
| Séfa 50 | P | 24.5 | ↑ | +29% |
| | (–) | 28.2 | | |

TABLE VII

ESTIMATE OF BEST ELEMENTS RATIOS IN LEAVES

| | PEANUT | OIL PALM |
|---|---|---|
| N : P | 20.0 | 16.0 |
| N : K | 3.5 | 3.0 |
| N : Ca | * | 3.0 |
| N : Mg | 5.0–6.0 | 9.0 |
| P : K | 0.20 | 0.20 |
| P : Ca | 0.10–0.15 | 0.20 |
| P : Mg | 0.3 | 0.5–0.75 |
| K : Ca | 1.0 | 2.0 |
| K : Mg | 2.0 | 2.0 |
| Ca : Mg | 2.0 | 2.0–2.5 |

* Difficult as yet to determine.

favorable treatment. There seems to be an optimum $N_L/P_L$ ratio around 20 (table VI).

Similar calculations have been made for other ratios, which are summarized in table VII.

Of course, these "optimal" ratios are still quite rough and rather indicate a range of ratios toward which favorable treatments will converge. Nevertheless, they may be useful in analyzing deficiencies. When a ratio found in a leaf sample differs greatly from these values, the divergence may be attributed to the numerator and/or the denominator, and the deficiency may thus become evident. For instance, in Thiès, $P_L/K_L = 0.582$ is about three times the "optimal" ratio. The figures show clearly that this is due to both a high $P_L$ and a low $K_L$, so the suggestion to apply $K_T$ is made. The same reasoning applies to Séfa 1951, where $P_L/K_L = 0.083$ (a value much too low) would point to an application of $P_T$. Experimental results confirm these predictions.

So, these values are a means of checking the conclusions drawn from the critical-level values. *They are especially useful in regions where no experimental fertilization data are available.*

*Correlations between elements:* The ten possible correlations between N, P, K, Ca, and Mg have been calculated. The most interesting ones can be seen in table VIII.

We have seen that the $N_L/P_L$ ratio is surprisingly constant in the oil palm, which is explained by the strong positive correlation found in all cases. Correlations are also positive in the peanut, although not so highly significant. On the other hand, $K_L$, $Ca_L$, and $Mg_L$ are linked together. The correlation between $K_L$ and $Ca_L$ or $K_L$ and $Mg_L$ are negative while the correlations between $Ca_L$ and $Mg_L$ are positive. Thus, the analyzed elements could be, broadly speaking, subdivided into two large classes: N and P could be termed the "protoplasmic class," while the other elements could be termed the "basic elements class."

It should be pointed out that the sum $K_L + Ca_L + Mg_L$ (table IV) for the peanut increases approximately as the yields (see fig. 3), and may be used to estimate the general soil exhaustion in available bases.

TABLE VIII

SIGN, VALUE AND SIGNIFICANCE OF THE CORRELATIONS BETWEEN PROTOPLASMIC ELEMENTS N, P AND BASIC ELEMENTS K, Ca, Mg, IN PEANUT AND OIL PALM EXPERIMENTS

| | NUMBER OF RESULTS | NP | | KCa | | KMg | | CaMg | |
|---|---|---|---|---|---|---|---|---|---|
| | | + | − | + | − | + | − | + | − |
| | | | | *Peanut* | | | | | |
| SENEGAL | | | | | | | | | |
| Thiès (1950) | 81 | 0.20 | | | 0.10 | | 0.21 | 0.30 ** | |
| Séfa (1950) | 81 | 0.10 | | | 0.64 ** | | 0.66 ** | 0.07 | |
| Séfa (1951) | 81 | 0.59 ** | | | 0.47 ** | | 0.74 ** | 0.65 ** | |
| Bambey (1951) | 81 | 0.07 | | | 0.65 ** | | 0.76 ** | 0.87 ** | |
| Bambylor (1951) | 81 | | 0.02 | 0.09 | | | 0.38 ** | 0.42 ** | |
| Louga (1951) | 40 | 0.68 ** | | | 0.08 | 0.22 | | 0.51 ** | |
| Guinguinéo (1951) | 40 | 0.18 | | | 0.32 * | | 0.43 ** | 0.66 ** | |
| FRENCH CONGO | | | | | | | | | |
| Loudima (1951) | 81 | 0.05 | | | 0.50 * | | 0.72 * | 0.22 * | |
| Inoni (1951) | 27 | 0.29 | | | 0.31 | | 0.24 | 0.08 | |
| | | | | *Oil Palm* | | | | | |
| DAHOMEY | | | | | | | | | |
| Pobé 2-year-old trees | 25 | 0.65 ** | | | 0.11 | | 0.62 ** | 0.39 | |
| 20-year-old trees | 48 | 0.22 * | | | 0.62 ** | | 0.83 ** | 0.55 ** | |
| IVORY COAST | | | | | | | | | |
| Dabou 20-year-old trees | 35 | 0.64 ** | | | 0.06 | | 0.31 | 0.05 | |
| FRENCH CONGO | | | | | | | | | |
| Sibiti 7-year-old trees | 21 | 0.52 * | | | 0.65 ** | | 0.48 * | 0.09 | |
| Etoumbi 20-year-old trees | 48 | 0.51 ** | | 0.09 | | | 0.02 | 0.62 ** | |

* Significant at 5%.
** Significant at 1%.

+ = positive correlations.
− = negative correlations.

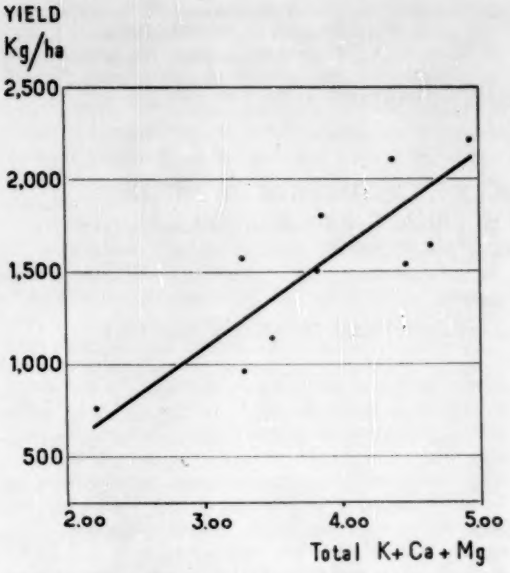



FIG. 3. Relation between the yield and the sum of the basic cation in the leaf of the peanut.

In the oil palm (table V) this sum is rather constant, around 2.0.

It is interesting to note that, although the favorable treatment may greatly increase $K_L$ (i.e., Pobé: $K_L$ goes up from 0.499 to 0.872), the sum is changed by only a very small amount (Pobé: from 2.08 to 2.12). This is well explained by the antagonisms between $Ca_L$, $Mg_L$, and $K_L$. At Dabou these antagonisms do not reach the significant level; the sum of the elements increased more (from 1.40 to 1.81) and there was concomitant increase of both $K_L$ and $Ca_L$. So, we see that when the sum does not reach 2.0 the antagonism between $K_L$ and $Ca_L$ or between $K_L$ and $Mg_L$ disappear.

## SUMMARY

Detailed results of a typical peanut experiment are reported to show the method used in foliar diagnosis. General results are given for the peanut and oil palm, and from these results critical levels of N, P, and K are approximated for both plants. Interrelations of elements are discussed from the standpoints of the ratios and correlations between N, P, K, Ca, and Mg. Correlations between N and P are generally positive; correlations between K and Ca or K and Mg are negative, while correlations between Ca and Mg are positive. In the oil palm such correlations may disappear when the sum K + Ca + Mg is lower than 2.0.

The elements thus far analyzed have been subdivided into two large classes: the "protoplasmic class" for N and P, and the "basic elements class" for K, Ca, and Mg.

This work has been made possible through the cooperation of the staff of the I.R.H.O., the members of the research stations in Africa, who conducted most of the experiments, and the chemists in Paris. Special acknowledgment is due to M. Ferrand, Conseiller Agronomique of the I.R.H.O., who initiated this research, and to Dr. A. Ulrich, who kindly agreed to read the manuscript.

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# THE RESPIRATION OF THE PEA PLANT. OXIDATION OF HEXOSE PHOSPHATE AND PENTOSE PHOSPHATE BY CELL-FREE EXTRACTS OF PEA LEAVES[1, 2, 3]

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Evidence for the presence of the Embden-Meyerhof-Parnas pathway in higher plants has accumulated rapidly in the last few years (23). The major outstanding difference reported between the glycolytic pathway in the green tissues of higher plants and in yeast, animal tissue and microorganisms is the presence in green tissues of a glyceraldehyde phosphate dehydrogenase linked not only to diphosphopyridine nucleotide but also to triphosphopyridine nucleotide (1, 13).

Preliminary evidence has also been gathering that an alternate pathway of glucose-6-phosphate metabolism characterized in yeast (6, 9, 10), animal tissue (11, 15, 16) and microorganisms (8, 12), and termed the "hexosemonophosphate shunt" may also be operating in leaves (3, 13), roots (13) and other tissues (5, 7) in higher plants.

The present investigation was undertaken to establish whether the enzymes of the "hexosemonophosphate shunt" are present in pea leaves and, if so, (1) whether the pathway is the same as in tissue of other organisms and (2) whether it is connected with the glycolytic pathway.

## Materials and Methods

Fructose-1,6-diphosphate (FDP), fructose-6-phosphate (F-6-P), and adenosine triphosphate (ATP) were purchased from the Schwarz Laboratories. Glucose-6-phosphate (G-6-P) and 6-phosphogluconate (6-PG) were prepared by the methods of Seegmiller and Horecker (21). Ribose-5-phosphate (R-5-P) was kindly supplied by Dr. R. DeMoss. Ribonic acid-5-phosphate prepared from ribose-5-phosphate by the hypoiodite oxidation procedure of Moore and Link (19) was kindly supplied by Dr. L. M. Paege. Coenzyme I (diphosphopyridine nucleotide, DPN) was obtained from the Schwarz Laboratories. Analysis showed that it contained 61% DPN. Coenzyme II (triphosphopyridine nucleotide, TPN) was prepared by the method of LePage and Mueller (17) and found to contain 31% Co II and to be free of Co I. TPN (80%) purchased from the Sigma Chemical Co. was also used. The barium salts of the phosphate esters were converted into potassium salts by dissolving in 1 *N* HCl, adding a slight excess of 10% $K_2SO_4$ and removing the resulting $BaSO_4$ by centrifugation. The supernatant solution was neutralized with 1 *N* KOH and diluted to make the final substrate concentration either 0.1 M or 0.05 M.

Preparation of Pea Leaf Extract: *Pisum sativum* variety Alaska (Rogers Brothers Seed Co., Idaho Falls, Idaho) was used throughout this investigation. The seeds were soaked overnight in tap water, then transplanted to tanks containing gravel in the greenhouse. Nutrient solution (14) was poured over the gravel once during the period of growth.

It is in order to note that cell-free preparations of leaves obtained from plants grown in the winter (November through March) were unsatisfactory for manometry, although solar radiation was supplemented with artificial light, total radiation being 300–800 fc.

Leaves of 11–15 day plants were used. A typical batch of plants had 4–5 sets of leaves, weighed about 2 gm, and disregarding the cotyledons, the weight was approximately evenly divided among the leaves, stems and roots. In general, about 70 gm of leaves, separated from the stems, were ground with an equal weight of acid-washed, cold, sharp sand and 20 ml of ice-cold 0.5 M $KHCO_3$ in a cold mortar. This operation and subsequent ones in the preparation of the cell-free extracts were carried out at 0–4°C. The extract was strained through 4 thicknesses of cheesecloth and centrifuged at 18,000 × g for 20 minutes. On occasion the supernatant material was passed through glass wool to remove fat particles. The solution had a dark green color, its volume was 50–75 ml and its pH varied from 6.8 to 7.5. The pH was brought to 7.2 with either ice-cold 1 *N* KOH or 2 *N* acetic acid. This preparation was used immediately or placed in the deep freeze until the next day. The frozen material when thawed the next morning formed a heavy precipitate which was removed by centrifugation at 18,000 × g for 20 minutes. The removal of this precipitate did not affect the activity of the extract. In some of the experiments, the extract was dialyzed 6

[1] Received March 16, 1953.
[2] A preliminary report was given at the meetings of the American Institute of Biological Science, Ithaca, N. Y., September, 1952.
[3] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

hours against 10 liters of 0.02 *N* $NaHCO_3$ or 0.02 *N* $NaHCO_3$ containing 47 mM cysteine · HCl. The pH of the dialyzing mixture was 7.5.

On occasion, extracts were prepared in the usual manner with 150 mg of sodium versenate (pH 7.4) added to protect the sulfhydryl-containing enzymes. Extracts prepared in this manner were inactive for manometry, presumably due to the removal of cofactors.

Oxygen uptake was determined manometrically at 37° with air as the gas phase. The main compartment of each Warburg vessel usually contained 1.0 ml of dialyzed enzyme solution, 0.1 ml of 0.2 M $MgCl_2$, 0.05 ml riboflavin (1 mg/ml), and 0.05 ml of 0.25 M ATP, or undialyzed enzyme solution, 1.0 ml, and water, 0.45 ml. The side arm contained 0.05 ml TPN solution (1 mg/ml) and 0.2 ml of 0.05 M substrate. The center well contained 0.2 ml 20% KOH as $CO_2$ absorbent or in experiments with cyanide a suitable KOH-KCN mixture. After the endogenous level had subsided (usually 60–90 minutes), the reaction was initiated by tipping in the TPN and substrate. For each point on the curves, duplicates were run.

The rate of reduction of TPN was followed at 340 mμ in 1 cm cells in a Beckman spectrophotometer, model DU, at 23–25°. The cell contained 1.5 ml veronal buffer, pH 8.0, 0.05 ml enzyme solution, 0.05 ml TPN (1 mg/ml) and 0.1 ml of 0.05 M substrate. Water was added to make a total volume of 3 ml. Blanks omitting TPN were used.

## Results

Figure 1 indicates that the cell-free preparation contains enzymes capable of oxidizing fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate. The following materials are not oxidized: glucose, gluconic acid, ribose, ribonic acid-5-phosphate, hydroxypyruvic acid and formic acid. When formic acid was used as substrate DPN was also added; however, it had no effect.

It is also apparent from figure 1 that the extract

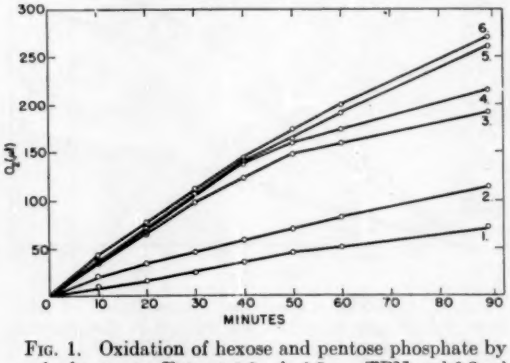


Fig. 1. Oxidation of hexose and pentose phosphate by pea leaf extract. Extract 1.0 ml; 0.5 mg TPN and 0.2 ml 0.05 M substrate. Total volume 1.7 ml; 1. endogenous; 2. 6-phosphogluconate; 3. glucose-6-phosphate; 4. fructose-6-phosphate; 5. ribose-5-phosphate; 6. fructose-1,6-diphosphate.

Table I

Effect of Boiled Pea Leaf Extract on the Oxidation of Glucose-6-phosphate by Pea Leaf Extract

| Reactants * | $O_2$ uptake (μL/110 min) |
|---|---|
| Undialyzed P.L.E.,† G-6-P, TPN | 232 |
| Dialyzed P.L.E., TPN | 67 |
| Dialyzed P.L.E., G-6-P | 42 |
| Dialyzed P.L.E., TPN, G-6-P + 0.2 ml B.P.L.E.‡ | 33 |
| Dialyzed P.L.E., TPN, G-6-P + 0.5 ml B.P.L.E. | 58 |
| Dialyzed P.L.E., TPN, G-6-P + 1.0 ml B.P.L.E. | 118 |

* 1.0 ml pea leaf extract dialyzed against $NaHCO_3$, 0.5 mg TPN, 0.2 ml 0.05 M glucose-6-phosphate. Total volume 2.5 ml.

† P.L.E.—pea leaf extract.

‡ B.P.L.E.—undialyzed pea leaf extract heated 2 minutes at 100°C.

contains the entire complement of enzymes necessary for the transfer of electrons from the substrate to oxygen. After dialysis, the pea leaf extract loses its capacity to oxidize hexose and pentose phosphate, however, the oxidizing activity of the dialyzed preparation is restored by the addition of pea leaf extract heated at 100°C for 2 minutes (table I). These heat-stable cofactors are also present in boiled yeast juice. Of the various likely cofactors tested, a mixture of TPN, ATP and riboflavin or riboflavin phosphate increases the oxygen uptake when any of the hexose or pentose phosphates is employed as substrate (tables II, III, IV).

Table II

Effect of Cofactors on the Oxidation of Fructose-1,6-diphosphate by Dialyzed Pea Leaf Extract

| Addition | $O_2$ uptake (μL/120 min) |
|---|---|
| FDP, riboflavin, ATP, TPN | 370 |
| Riboflavin, ATP, TPN | 112 |
| FDP, ATP, TPN | 201 |
| FDP, riboflavin, TPN | 199 |
| FDP, riboflavin, ATP | 117 |

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

With some extracts the rate of oxidation of FDP

Table III

Effect of Adenosine Triphosphate on the Oxidation of Fructose-1,6-diphosphate by Pea Leaf Extract

| Micromoles of ATP | $O_2$ uptake (μL/50 min) |
|---|---|
| 0 | 50 |
| 5 | 90 |
| 10 | 117 |
| 15 | 117 |
| 20 | 132 |
| 30 | 125 |

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

TABLE IV

EFFECT OF RIBOFLAVIN AND RIBOFLAVIN-5-PHOSPHATE ON THE OXIDATION OF FRUCTOSE-1,6-DIPHOSPHATE BY PEA LEAF EXTRACT

| MICROGRAMS | | $O_2$ UPTAKE ($\mu$L/360 MIN) |
|---|---|---|
| RIBOFLAVIN-5-P | RIBOFLAVIN | |
| .. | ... | 259 |
| 1.3 | ... | 256 |
| 2.6 | ... | 274 |
| 6.5 | ... | 315 |
| .. | 1.0 | 271 |
| .. | 2.0 | 304 |
| .. | 5.0 | 389 |
| .. | 10.0 | 400 |
| .. | 20.0 | 410 |

See methods section for protocol. Extract dialyzed against $NaHCO_3$.

is higher than with other substrates. Also, when FDP oxidation is carried out with preparations in which the triosephosphate dehydrogenase was presumably inactivated by addition of 0.01 M iodoacetamide (fig. 2) or by dialysis against 0.02 M $NaHCO_3$, the rate of oxygen uptake was decreased to the level obtained with G-6-P. This indicates the FDP is being metabolized via two pathways.

Table V indicates that the leaf extract also oxidizes

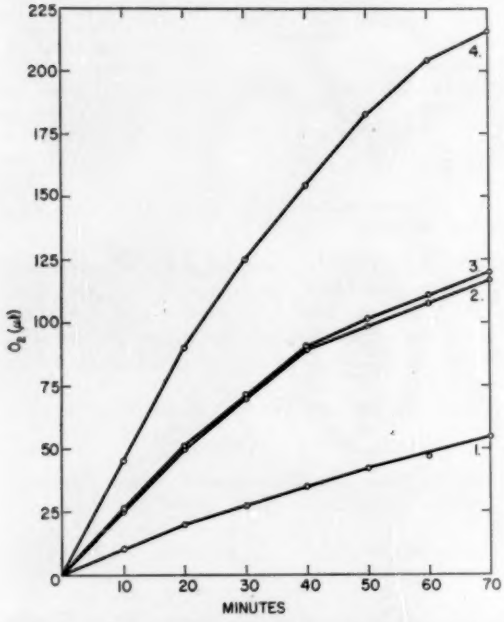


FIG. 2. Effect of iodoacetamide on fructose-1,6-diphosphate oxidation. Pea leaf extract 1.0 ml; 0.5 mg TPN and 0.2 ml 0.05 M substrate. Total volume 1.7 ml; 1. endogenous; 2. glucose-6-phosphate; 3. fructose-1,6-diphosphate and iodoacetamide—final concentration 0.01 M; 4. fructose-1,6-diphosphate.

TABLE V

DPN-LINKED TRIOSEPHOSPHATE DEHYDROGENASE IN PEA LEAF EXTRACT

| COMPONENTS OF SYSTEM | $O_2$ UPTAKE ($\mu$L/90 MIN) |
|---|---|
| Complete system * ..... | 180 |
| – arsenate ..... | 60 |
| – ATP ..... | 30 |
| – FDP ..... | 30 |
| – riboflavin ..... | 140 |
| – NaF ..... | 190 |
| + iodoacetamide 0.0025 M (final conc.) .... | 140 |
| + iodoacetamide 0.01 M (final conc.) ..... | 100 |

* Complete system. Extract dialyzed against $NaHCO_3$-cysteine, 1.0 ml; sodium arsenate 36 $\mu$M; sodium fluoride 60 $\mu$M; FDP 10 $\mu$M; ATP 12.5 $\mu$M; riboflavin 0.13 $\mu$M; DPN 0.17 $\mu$M; cysteine 8 $\mu$M. Total volume 2.5 ml.

FDP via a DPN-triosephosphate dehydrogenase. Cofactor requirements (DPN and arsenate) are the same as for the DPN-triosephosphate dehydrogenase isolated from tissues of other organisms. On the other hand, the rate of oxidation of FDP is increased by only a small amount on the addition of arsenate (fig. 3) when TPN is used in place of DPN.

Coenzyme specificity was determined by following the reduction of DPN and TPN spectrophotometrically at 340 m$\mu$. The results (fig. 4) show a reduction of TPN by FDP, F-6-P, G-6-P, 6-PG, and R-5-P indicating the presence of dehydrogenases. In the presence of arsenate, FDP and R-5-P reduce DPN. None of the dehydrogenases is active with DPN alone. As indicated in figure 4, a lag of 30–45 seconds in TPN reduction occurs when R-5-P is substrate.

EFFECT OF ENZYME INHIBITORS: Cyanide (0.01 M) produces a 30% increase in glucose-6-phosphate dehydrogenase activity and a slight increase in the 6-phosphogluconic acid dehydrogenase. The rate of $O_2$

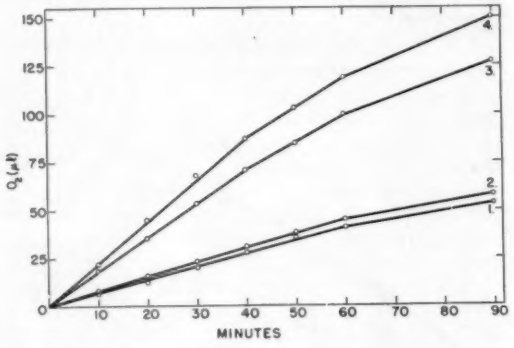


FIG. 3. Effect of arsenate on the oxidation of fructose-1,6-diphosphate by pea leaf extract in the presence of TPN. Extract dialyzed against $NaHCO_3$-cysteine 1.0 ml; 0.5 mg TPN; riboflavin 0.13 $\mu$M; ATP 12.5 $\mu$M. Total volume 2.0 ml; 1. endogenous; 2. endogenous, sodium arsenate 36 $\mu$M; 3. fructose-1,6-diphosphate 10 $\mu$M; 4. fructose-1,6-diphosphate 10 $\mu$M, sodium arsenate 36 $\mu$M.

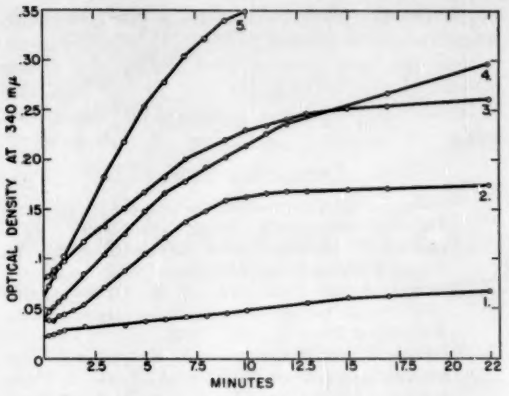


FIG. 4. Reduction of TPN by hexose and pentose phosphate. For details, see methods section. 1. 6-phosphogluconate; 2. ribose-5-phosphate; 3. fructose-1,6-diphosphate and 0.01 M iodoacetamide; 4. fructose-6-phosphate; 5. glucose-6-phosphate.

uptake is either not affected or slightly increased with any of the hexose phosphates (fig. 5) as substrates. These facts seem to indicate cyanohydrin formation and not inhibition of catalase as a possible explanation of increased $O_2$ uptake. Inhibition of catalase would result in a twofold increase in $O_2$ uptake.

*Azide and Fluoride:* Azide (0.01 M) and fluoride (0.01 M) do not affect the activity of any of the dehydrogenases or the $O_2$ uptake. The pH of the mixtures was 6.8 to 7.0 when azide was used.

*Iodoacetamide:* Iodoacetamide (0.01 M) did not inhibit glucose-6-phosphate dehydrogenase or 6-phosphogluconic acid dehydrogenase. It did inhibit (see fig. 2) the $O_2$ uptake when FDP was employed as substrate by approximately 50%. The poison did not inhibit the $O_2$ uptake when G-6-P or 6-PG were oxidized.

*Indoleacetic Acid:* High concentrations (0.01 M) did not affect the $O_2$ uptake when FDP, F-6-P, G-6-P, 6-PG or R-5-P was employed as substrate.

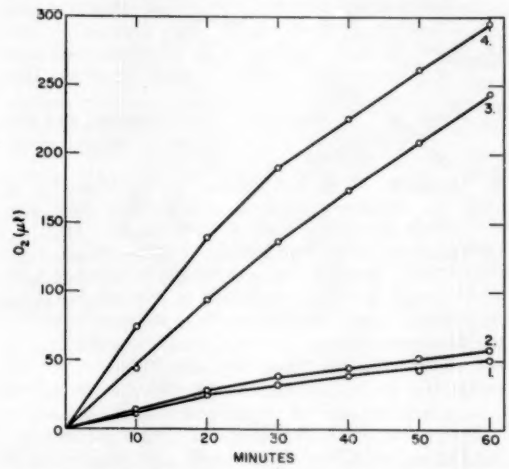


FIG. 5. Effect of cyanide on the oxidation of fructose-1,6-diphosphate. Pea leaf extract 1.0 ml; 0.5 mg TPN. Total volume 1.7 ml; 1. endogenous; 2. endogenous, cyanide—final concentration 0.01 M; 3. 0.2 ml 0.05 M fructose-1,6-diphosphate; 4. 0.2 ml 0.05 M fructose-1,6-diphosphate, cyanide—final concentration 0.01 M.

## DISCUSSION

The present work establishes the existence in the pea leaf of a pathway which has been designated as "the hexosemonophosphate shunt." As in animal tissue and yeast, all of the dehydrogenases involved are TPN-specific and are not inhibited by fluoride and iodoacetamide.

Inhibition of $O_2$ uptake in the presence of iodoacetamide is apparently due to a poisoning of the DPN- or TPN-linked glyceraldehyde phosphate dehydrogenase. Inhibition (50%) of the rate of fructose-1,6-diphosphate oxidation by iodoacetamide demonstrates that in these preparations about half of the ester is being oxidized via the "hexosemonophosphate shunt" pathway and the remainder via the Embden-Meyerhof-Parnas pathway. James and Beevers (17) had reported a partial inhibition of the oxidation of fructose-1,6-diphosphate by cell-free extracts of the Arum spadix in the presence of iodoacetamide. Stumpf (24) found no inhibition of fructose-1,6-diphosphate oxidation by iodoacetamide; however, the preparations were made by use of the Waring blender which may have inactivated the triosephosphate dehydrogenase.

The data indicate (table V) that arsenate is needed as a cofactor in fructose-1,6-diphosphate oxidation when DPN is used as electron acceptor. This requirement is similar to that of the DPN-linked triosephosphate dehydrogenase isolated from pea seeds (22). However, arsenate has little effect on fructose-1,6-diphosphate oxidation when TPN is added to the extract although the inhibition of $O_2$ uptake caused by iodoacetamide indicates the presence of triosephosphate dehydrogenase. The reduction of TPN with fructose-1,6-diphosphate as substrate and the absence of an arsenate effect on the reduction has been reported (3, 5). This observation has been confirmed in this laboratory. However, these same extracts require arsenate as cofactor when glyceraldehyde-3-phosphate is employed as substrate in place of fructose-1,6-diphosphate.[4] Thus, the possibility exists that fructose-1,6-diphosphate can be converted to triosephosphate and be metabolized by an alternative pathway which does not include the arsenate-requiring triosephosphate dehydrogenase.

Ribose-5-phosphate which arises from the decarboxylation of 6-phosphogluconate (5, 15) is readily oxidized by the pea leaf extract; however, the same extract is not able to oxidize ribonic acid-5-phosphate. This behavior is similar to that found in rat and kidney extracts (11). After a short lag, ribose-5-phosphate reduces TPN (fig. 4). This lag is presumably due to a slow conversion of ribose-5-phosphate to

[4] Gibbs. Unpublished data.

ribulose-5-phosphate (15, 20). Present evidence indicates that the ketopentose sugar incubated with spinach leaf preparations yields sedoheptulose phosphate and triosephosphate (4). The latter can reduce TPN via the TPN linked triosephosphate dehydrogenase of the Embden-Meyerhof glycolysis pathway.

Lack of inhibition by cyanide when hexose phosphate is substrate together with the requirement of microgram quantities of riboflavin or riboflavin-5-phosphate and milligram quantities of adenosine triphosphate suggests a flavin-type respiratory system with a phosphorylated riboflavin as cofactor. A similar observation was reported for the Arum spadix (17). Flavin-adeninedinucleotide with adenosine triphosphate has also been reported to stimulate respiration in pea leaf extracts (24).

## Summary

1. Cell-free extracts of 11–15-day pea leaves oxidize fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate, ribose-5-phosphate in the presence of triphosphopyridine nucleotide. Glucose, gluconic acid, ribose, ribonic acid-5-phosphate and formic acid did not accelerate the $O_2$ uptake of the leaf extract.

2. The oxidation of glucose-6-phosphate, 6-phosphogluconate and ribose-5-phosphate indicates the presence in leaf tissue of a "hexosemonophosphate shunt" which closely resembles that found in animal tissue and yeast. The oxidations are TPN-specific and not inhibited by iodoacetamide or fluoride.

3. In some extracts, the $O_2$ uptake with fructose-1,6-diphosphate is approximately double that with glucose-6-phosphate. On addition of 0.01 M iodoacetamide to such extracts or to dialyzed extracts, the rate of $O_2$ uptake becomes equal for the two substrates.

4. The failure of the pea leaf extract to oxidize ribonic acid-5-phosphate while oxidizing ribose-5-phosphate indicates that ribose-5-phosphate can be metabolized by conversion to compounds of the Embden-Meyerhof glycosis pathway.

5. Dialyzed leaf extracts lose the ability to oxidize hexose or pentose phosphate. This ability can be restored with microgram quantities of riboflavin or riboflavin-5-phosphate in addition to milligram quantities of adenosine triphosphate. Added boiled pea juice or boiled yeast extract produce the same effect.

6. Leaf extracts oxidize fructose-1,6-diphosphate in the presence of DPN provided arsenate is present. When TPN is substituted for DPN, arsenate stimulates the $O_2$ uptake only slightly.

7. Cyanide (0.01 M) does not inhibit the oxidation of hexose phosphate and may accelerate the early stages of oxidation. It does inhibit ribose-5-phosphate oxidation.

8. Azide (0.01 M) or fluoride (0.01 M) does not affect the $O_2$ uptake of these substrates.

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# EFFECTS OF SHADE AND PARTIAL DEFOLIATION ON CARBOHYDRATE LEVELS AND THE GROWTH, FRUITING AND FIBER PROPERTIES OF COTTON PLANTS[1]

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Carbohydrate levels in cotton plants during the fruiting period were reduced: 1. by growing the plants under muslin shades and 2. by cutting away half of each new leaf. The effects of these treatments were studied and are discussed in terms of 1. light in relation to growth of the cotton plant, 2. partial defoliation and growth, 3. relative fruitfulness (bolls per 100 gm of fresh stems and leaves) and 4. changes in boll and fiber properties.

The foregoing experiments were conducted along with others (partial defruiting and drought) that increased carbohydrate levels. Both of these treatments (4) increased fiber strength. Partial defruiting increased slightly and drought decreased: seed cotton per boll, fiber length, weight per inch and percent of mature fibers; but between five varieties of cotton it was found that those operating at the lowest carbohydrate levels had the strongest fibers, i.e., strength was evidently correlated with enzymatic and other growth factors that furthered carbohydrate utilization. As a further background for the results of this paper, it has been found that boll shedding is not commonly related to the carbohydrate or nitrogen levels in the plant (5).

The shade experiment (light intensity reduced to 32% of full exposure by muslin sheeting) was repeated at a high, intermediate and low level of water supply and was started at about the time of first flowers. References were made to the findings of others on the effects of light intensities in a previous paper (6). In the partial defoliation (half-leaf) experiment, one-half of each of the previous leaves and of each subsequent new leaf was removed weekly starting also at the time of first flowers. The work was conducted at the U. S. Cotton Field Station, Shafter, California, with the Acala p18 variety of cotton. Consideration is given in the discussion to evidence bearing on the auxin relations involved in the fruiting of the cotton plant.

[1] Received March 26, 1953.

## Methods

The two experiments, shade and half-leaf, were each laid out in four blocks with the control and treated plots alternating in irrigation borders 320 feet long; different irrigation borders were used for the two tests. Twelve rows of cotton with the plants spaced 12 inches apart were used for the shade experiment and four rows of similarly spaced plants for the half-leaf (partial defoliation) experiment. The four shades were 25 feet long; plants in the outside rows under the shades as well as the three end plants under and outside of the shades were not used for the measurements. The three levels of water supply—heavy, medium and light—were supplied by irrigating: a, both sides of three rows weekly, b, only one side of three rows weekly and, c, one side of each of three rows every two weeks. One-half of each control plot was to the east and one-half to the west of each of the shades. The analyses of variance for *yields* assume randomization which would have been difficult or impractical in the instance of the irrigation treatments.

Twelve plants were sampled at three times during the flowering period for carbohydrate analyses in both experiments. At each collection half of the plants were harvested in the late morning and half in the early afternoon; the order of collection was reversed between morning and afternoon and between successive days. In the half-leaf experiment the sampling dates were July 14, 15, 29, and 30 and August 11 and 12, tissues from three plants in each treatment being collected each time and the twelve then combined as single samples. In the shade experiment six plants were sampled in the late morning and six in the early afternoon as above but during the single days: July 16 and 31 and August 13. The leaf samples (48 leaves per sample) were from the middle third of the main stalk. The middle third of the main stalk was used for the stem samples. The dated bolls were from the plants sampled.

Measurements of height and number of nodes and

of fresh plant weight and of number of bolls per plant, from which relative fruitfulness (bolls per 100 gm of fresh stems and leaves) is calculated were made in the half-leaf experiment on August 7 using 48 plants from each treatment. Plants that had retained their terminal buds intact and that were free of symptoms of Verticillium wilt were selected for these measurements. Similar data were obtained in the shade experiment on August 21 but only 24 plants from each treatment were measured. The relative fruitfulness values are calculated plant by plant and the means shown in the tables. The introduction of bias by using selected rather than random plants will be referred to later in the text.

A one-hundred-boll sample of seed cotton was collected from each of the two treatments of the half-leaf experiment on each of three dates. The data in table IV represent the mean values with the analysis of variance based on dates rather than replicates. In the shade experiment only a single 100-boll sample from each of the six treatments was collected on October 19 with 25 of these bolls from each replicate. It was intended that these 25-boll samples be kept separate but through a misunderstanding they were combined; this having been the case no basis existed for analyses of statistical significance. This difficulty has been overcome, largely, by comparing the fiber-property results of 1947 with those from a similar experiment conducted in 1946; otherwise the 1946 data are not tabulated for the reason that the DDT preparation used to control insects in 1946 was not effective and the plants were rank growing and poorly fruited. The effects of shade on fiber properties in the two years show differences from the control treatments that are in the same direction in each comparison.

The methods used in the carbohydrate and nitrogen analyses were cited in an earlier paper (5).

Light-intensity was measured with a Weston photometer. Results of light-intensity measurements at various heights above the ground among cotton plants are reported both for 1946 and 1947.

The fiber property measurements were all made through the courtesy of the Cotton Branch of the Production and Marketing Administration.

## Experimental

Shades: *Self-shading in Cotton Plantings:* Before reviewing the effects of shading on carbohydrates and on growth and development of the cotton plants, attention will be given to a limited series of light-intensity measurements taken at successive heights among cotton plants grown under various conditions. The data are expressed as percentages of the vertical intensities measured above the tops of the plants; each value is the mean of five readings in the case of irrigated cotton and of three readings in the 1946 dry cotton. Although the data of table I do not permit conclusions with respect to the progressive reductions in light intensity by self-shading that occur with the advance of the summer, they serve to bring out other points pertinent to the discussions.

Vertical light intensities measured on July 8, 1946, between 12:20 and 12:40 P.M. averaged 8860 fc in the open and 2036 fc under the shades. On August 19 the intensities between 12 and 12:40 P.M. averaged 7630 fc and during the subsequent half-hour period 6900 fc. The measurements of 1947 were made on August 26; just after noon the intensities averaged 5800 fc in the open and 1856 fc under the shades whereas later in the hour during the successive-height measurements the average intensity above the plants in the open was 5280 fc. At Shafter the light intensity often varies extensively from moment to moment with changes in the density of the variable haze.

The muslin shades used in 1946 reduced light intensities above the plants to 23%. In 1947 the reduction was to 32%.

The mean light intensity on the ground beneath the

Table I

Mean Unsmoothed Percentages of Vertical Sky Illumination Found in Mid-August at Successive Heights from the Ground Among Cotton Plants Under Various Conditions at Shafter, Calif., in 1946 (Plants Two Feet Apart) and 1947 (Plants One Foot Apart) and Approximate Percentage Light Transmissions to the Level of Half the Plant Height

| | Open, irrigated | | | | | | Open, dry | | | Shades, irrigated | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1946 | | | 1947 | | | 1946 | | | 1946 | | | |
| | *Rows* | *Centers* | *Mean* | *Rows* | *Centers* | *Mean* | *Rows* | *Centers* | *Mean* | *Rows* | *Centers* | *Mean* | *Of open* |
| Height of reading, cm 150 ... | 100 | 100 | 100 | ... | ... | .... | ... | ... | .... | 100 | 100 | 100 | 23 |
| " " " " 120 ... | 43 | 81 | 62 | 100 | 100 | 100 | 100 | 100 | 100 | 46 | 49 | 47 | 11 |
| " " " " 90 ... | 16 | 50 | 33 | 56 | 82 | 69 | 99 | 100 | 99 | 27 | 30 | 28 | 6 |
| " " " " 60 ... | 11 | 43 | 27 | 14 | 37 | 26 | 93 | 97 | 95 | 15 | 18 | 17 | 4 |
| " " " " 30 ... | 7 | 10 | 9 | 8 | 23 | 16 | 59 | 96 | 78 | 10 | 11 | 11 | 3 |
| " " " ground ... | 1 | 3 | 2 | 2 | 4 | 3 | 35 | 91 | 63 | 8 | 9 | 8 | 2 |
| Plant height, cm ..... | ... | ... | 160 | ... | ... | 110 | ... | ... | 95 | ... | ... | 163 | .... |
| Approx. light at ½ height, % | ... | ... | 34 | ... | ... | 27 | ... | ... | 84 | ... | ... | 24 | 6 |
| Vertical, sky at time, fc .... | ... | ... | 7630 | ... | ... | 5280 | ... | ... | 6900 | ... | ... | 1500 | (7630) |

irrigated cotton was in each comparison less than 5% of that at the tops of the plants. In wide-spaced dry cotton of 1946, 63% of light reached the ground. Such a high penetration in wide-spaced cotton is perhaps unusual because the drouth treatment was so severe that it repressed the extensive development of vegetative branches normally occurring in wide-spaced cotton; these unirrigated plants wilted daily after July 12. Only 2% of the incident radiation above the shades reached the ground under the shaded plants of 1946; comparable measurements were not made in 1947.

The percentages of light reaching downward to the half-height of the plants are reported along the bottom of table I for each of the treatments. These data are derived approximately by smoothing and interpolating the mean values in the column above. In the irrigated cotton of 1946 and 1947 the respective light values at half-height are 34 and 27%. Under the 1946 shades the half-height value is 6% of the full outside illumination. Counts have shown (6) that approximately half the leaves of the cotton plant originate from branches below and half from those above the middle main-stalk node. On the other hand, the middle node of the main stalk (cotyledons taken as the zero node) has been found in a few counts to be located at about 40% of the plant height; the upward slope of the branches compensates in some measure for this discrepancy. Also, many of the leaves in the dark cone at the base of the cotton plant have undoubtedly passed their photosynthetic prime by mid-August.

Assuming that 6,000 fc may be a creditably high clear-day value for the 4-hour midday average light intensity in August it becomes of interest to convert some of the foregoing percentage-transmission values into fc intensities at the half-heights of the cotton plants. With a 30% transmission of light to the half-height of the irrigated plants in the open, the mean four-hour intensity would be 1800 fc. The foregoing 1800 fc intensity (half of the leaves receiving more and half less) would thus represent a mid-August midday maintenance intensity for carbohydrate synthesis to support boll development and respiration. After mid-August few new bolls are set and there is little additional vegetation development.

Investigators have found that for other plants (see later references) some require more and some less than the foregoing 1800 fc half-height intensity for full photosynthetic activity. Under the shades in 1946 the half-height intensity was 6% of the full vertical intensity; on the basis of a 6,000 fc illumination this would be only 360 fc as a mean maintenance intensity during the brightest four hours. Heinecke and Childers (9) note finding only 40 fc in the heart of a four-year-old apple tree when the outside intensity was 8100 fc.

The leaves of the plants under the shades were larger than those in the open but they were also thinner. Measurements made in 1946 of the lengths of midveins of the mainstalk leaves gave a mean value of 14.1 cm in the open versus 16.4 under the shades; in terms of leaf area the latter value would represent an increase in the order of 30%. In 1947 a few measurements were made of the percentages of light transmission through leaves collected from comparable positions on plants in the open and under the shades. For this comparison the leaf blades were held against the face of the photometer target and exposed vertically; the readings so obtained are related to full exposures as percentage-transmission values:

Leaves from the open 4.4, 5.6 and 6.0: mean 5.3% transmission.

Leaves from the shades 7.6, 8.2 and 12.9: mean 9.6% transmission.

*Carbohydrates and Nitrogen Responses:* The moisture contents of each of the plant parts examined was greater—at corresponding water-supply levels—under the shades than in the open. Throughout the data there is evidence of marked interactions between water supply and light in their effects on carbohydrate levels. To interpret the effects of the treatments on plant reactions in terms of either light intensity, moisture supply, tissue moisture or carbohydrate levels alone would be open to question since each must influence through interactions the paths of cause and effect. Undoubtedly both light and moisture supply influence auxin levels and the rates of carbohydrate utilization and photosynthesis. It may be noted in table II that both the stems and 14-day-old bolls had higher percentages of total carbohydrate at the low moisture under the shades than at high moisture in the open.

In terms of the factorial summaries of table II, reducing by shades the above-plant vertical light intensity to 32% of the outside exposure reduced the sum of sugars and starch in the leaves by 24%, in the stems by 38% and in the 14-day-old bolls by 8%. These relative reductions seem fairly reasonable: a moderate reduction in the rate of photosynthesis in the leaves would be expected to have a somewhat greater effect on the accumulation of sugars and starch in the storage tissues of the stems. On the other hand, for a normal growth and development of cotton bolls a rather even balance might be expected—temperature and moisture being the same—between the supply potential in the boll and the rate of utilization in cell-wall and protein formation. The shade plants carried only about half as many bolls as the plants in the open and the effect on photosynthesis was therefore much greater than is indicated by the carbohydrate accumulations in the leaves and stems.

Sucrose and starch in the leaves and stems were reduced substantially by the shades whereas the hexose sugars were not affected. Drouth, on the other hand, increased hexose sugars in the leaves but was without effect on sucrose and had little effect on starch (4). All three constituents were increased in the stems by drouth. Though lacking statistical significance, the shades rather consistently reduced hexose sugars and starch in the young bolls; water deficiency increased both. Little sucrose is found in cotton bolls.

Slightly lower total N was found in the leaf, stem

TABLE II

CARBOHYDRATES AND NITROGEN IN LEAVES, STEMS AND 14-DAY-BOLLS IN THE OPEN AND UNDER MUSLIN SHADES AT THREE MOISTURE LEVELS. THE DATA REPRESENT THE MEANS, EXPRESSED ON PERCENTAGES OF FRESH WEIGHT OF SAMPLES COLLECTED JULY 16, 31 AND AUGUST 14, 1947, SHAFTER, CALIFORNIA. THE DRY WEIGHTS OF SAMPLES—PER LEAF, ½ STEM AND BOLL—ARE ALSO SHOWN

| | MOISTURE | HEXOSE | SUCROSE | STARCH | SUM | TOTAL NITROGEN | DRY SAMPLE PER PIECE |
|---|---|---|---|---|---|---|---|
| LEAVES | | | | | | | *gm* |
| Open—heavy irri. | 77.9 | 0.08 | 0.19 | 0.42 | 0.69 | 0.70 | 1.12 |
| " medium " | 77.0 | .10 | .14 | .45 | .69 | .71 | 1.10 |
| " light " | 74.7 | .15 | .15 | .51 | .81 | .70 | .95 |
| Shade—heavy irri. | 81.0 | .10 | .07 | .38 | .55 | .62 | .84 |
| " medium " | 80.2 | .10 | .06 | .37 | .53 | .65 | .95 |
| " light " | 78.3 | .13 | .09 | .34 | .56 | .68 | .91 |
| Means—factorial | | | | | | | |
| Open | 76.5 | .11 | .16 | .46 | .73 | .70 | 1.06 |
| Shade | 79.8 | .11 | .07 | .36 | .54 | .65 | .90 |
| LSD at 5% | .92 | .03 | .04 | .09 | .08 | .07 | .06 |
| Heavy irri. | 79.4 | .09 | .13 | .40 | .62 | .66 | .98 |
| Medium " | 78.6 | .10 | .10 | .41 | .61 | .68 | 1.03 |
| Light " | 76.5 | .14 | .12 | .42 | .68 | .69 | .93 |
| LSD at 5% | 1.1 | .05 | .07 | .11 | .10 | .09 | .07 |
| STEMS | | | | | | | |
| Open—heavy irri. | 74.0 | .35 | .64 | .53 | 1.52 | .22 | 5.05 |
| " medium " | 70.8 | .40 | .81 | .74 | 1.95 | .25 | 3.74 |
| " light " | 67.9 | .64 | 1.15 | .96 | 2.75 | .28 | 3.42 |
| Shade—heavy irri. | 76.7 | .27 | .50 | .28 | 1.05 | .18 | 3.87 |
| " medium " | 72.5 | .33 | .58 | .26 | 1.17 | .24 | 3.62 |
| " light " | 69.7 | .48 | .85 | .36 | 1.69 | .23 | 3.32 |
| Means—factorial | | | | | | | |
| Open | 70.9 | .46 | .87 | .74 | 2.07 | .25 | 4.08 |
| Shade | 73.0 | .36 | .64 | .30 | 1.30 | .22 | 3.60 |
| LSD at 5% | 3.8 | .08 | .21 | .21 | .29 | .04 | .62 |
| Heavy irri. | 75.4 | .31 | .57 | .40 | 1.28 | .20 | 4.46 |
| Medium " | 71.6 | .36 | .70 | .50 | 1.56 | .24 | 3.68 |
| Light " | 68.8 | .56 | 1.00 | .66 | 2.22 | .26 | 3.37 |
| LSD at 5% | 4.6 | .09 | .26 | .26 | .35 | .05 | .75 |
| 14-DAY-OLD BOLLS | | | | | | | |
| Open—heavy irri. | 87.0 | 1.48 | .02 | 1.29 | 2.79 | .26 | 2.38 |
| " medium " | 85.2 | 1.64 | .01 | 1.57 | 3.22 | .30 | 2.68 |
| " light " | 84.7 | 1.62 | .01 | 1.62 | 3.25 | .30 | 2.62 |
| Shade—heavy irri. | 87.7 | 1.52 | .01 | 1.22 | 2.75 | .27 | 1.88 |
| " medium " | 87.2 | 1.42 | .02 | 1.19 | 2.63 | .28 | 2.12 |
| " light " | 86.0 | 1.50 | .05 | 1.34 | 2.89 | .29 | 2.10 |
| Means—factorial | | | | | | | |
| Open | 85.6 | 1.58 | .01 | 1.49 | 3.08 | .29 | 2.56 |
| Shade | 87.0 | 1.48 | .02 | 1.25 | 2.75 | .28 | 2.03 |
| LSD at 5% | 1.0 | .17 | .03 | .28 | .38 | .02 | .26 |
| Heavy irri. | 87.4 | 1.50 | .02 | 1.26 | 2.78 | .26 | 2.13 |
| Medium " | 86.2 | 1.53 | .01 | 1.38 | 2.92 | .29 | 2.40 |
| Light " | 85.4 | 1.56 | .02 | 1.48 | 3.06 | .30 | 2.36 |
| LSD at 5% | 1.2 | .21 | .04 | .50 | .46 | .03 | .32 |

and young boll tissues under the shades than in the open but in no instance were the differences significant. Measurements were made also of soluble and insoluble N but these values are omitted from the tables since the N distributions in the two fractions varied no more than did total N. Significantly more N was found in the stems and in the bolls at low than at high moisture; a similar trend is evident also in the leaves.

*Vegetative and Reproductive Development:* The cotton plant is unusually responsive to its environment in the effects on such characters as height,

weight, number of main-stalk nodes, fruiting branch nodes, leaf size and fruitfulness. But in any field planting of cotton all of these responses are influenced by irregularities in spacing, soil variability, disease, etc., and, particularly during early growth, by damage to the terminal buds by thrips. When the apical bud is destroyed, two or more vegetative branches develop to replace the main stalk; the fruiting branches on these limbs are shorter than on undamaged plants and fruiting is delayed. Occasional plants at Shafter exhibited symptoms of Verticillium wilt. Some selection of plants is accordingly desirable if representive mean values for normal plants are to be obtained; but such selections without bias is quite difficult. The selections were made with the reservation that proof of representativeness would be provided by comparing the relative number of developing bolls on the selected plants in the open and under the shades with relative yields of the treatments when the cotton was picked. Rather creditable comparisons resulted in the instance of the heavily irrigated plants in the open and under shades but in the medium and light irrigation treatments consistently good agreements did not result. The heavily irrigated plants at the time of the plant measurements had 49% as many bolls per plant in the shade as in open; the mean yield of seed cotton was 53% as great in the shade as in the open. Due to the inconsistencies at the intermediate and low moisture levels the following tabulation of the results is confined to the plants grown at the high moisture level:

| | Open | Shade |
|---|---|---|
| Yield of seed cotton, kilos per plot .. | 6.23 | 3.32 |
| Fresh leaves plus stems, gm ..... | 382 | 332 |
| Developing bolls per plant, number | 22.5 | 11.0 |
| Relative fruitfulness ..... | 6.0 | 3.3 |
| Fresh weight tops including bolls, gm | 846 | 561 |
| Height, cm ..... | 110 | 115 |
| Main stalk nodes, number ..... | 25.8 | 24.6 |
| Length longest fruiting branch, cm .. | 40 | 45 |
| Nodes longest fruiting branch, number | 4.8 | 4.0 |
| Vegetative branches over 20 cm long, number ..... | 1.2 | 1.2 |

Reducing light intensity to 32% of that above the shades lowered, under heavy irrigation, the fresh weight of leaves plus stems by only 14% but, in contrast, the number of bolls per plant was reduced to one-half. This specificity of light intensity in the partition of growth between vegetative and fruiting activities is in accord with the results of an earlier experiment (6) in which the plants were grown in sand-culture pots; the behavior of cotton plants under shades in a winter greenhouse was compared with similarly treated plants in pots outdoors in the summer. In that experiment low light reduced the fresh weight of leaves plus stems by 13% (means of 4 and 16 mE/l of $NO_3$ in sand cultures) and the number of bolls per plant was reduced from 22.9 to 11.3. Although the methods and conditions of that and the present experiments were very different, the values for plant weights and number of bolls are almost identical. The relative fruitfulness of the plants (bolls

TABLE III

YIELDS AND BOLL AND FIBER PROPERTIES AS INFLUENCED BY SHADE AND MOISTURE SUPPLY IN 1947 AND BY SHADE AT HIGH MOISTURE IN 1946

| TREATMENT | YIELD SEED COTTON | SEED COTTON PER BOLL | SEED INDEX | LINT INDEX | FIBER PROPERTIES | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | LENGTH UPPER HALF | TENSILE STRENGTH | WT PER INCH | MATURE |
| | *relative* | *gm* | *gm* | *gm* | *inches* | *relative* | *μg* | *%* |
| 1947 | | | | | | | | |
| Open—heavy irri. ..... | 6.2 | 7.3 | 13.2 | 9.1 | 1.07 | 6.3 | 3.8 | 81 |
| " medium " ..... | 5.5 | 7.5 | 13.1 | 9.6 | 1.04 | 6.4 | 4.2 | 80 |
| " light " ..... | 3.5 | 6.1 | 11.8 | 8.6 | 1.05 | 6.3 | 4.2 | 84 |
| Shade—heavy irri. .... | 3.3 | 6.6 | 12.7 | 8.6 | 1.10 | 5.6 | 3.7 | 76 |
| " medium " .... | 3.4 | 7.4 | 12.9 | 8.7 | 1.09 | 5.8 | 4.0 | 78 |
| " light " .... | 2.3 | 7.3 | 11.7 | 8.0 | 1.07 | 5.7 | 4.0 | 70 |
| Factorial summary | | | | | | | | |
| Light: | | | | | | | | |
| Open ..... | 5.1 | 7.0 | 12.7 | 9.1 | 1.05 | 6.3 | 4.1 | 82 |
| Shade ..... | 3.0 | 7.1 | 12.4 | 8.4 | 1.09 | 5.7 | 3.9 | 75 |
| LSD 5% ..... | .9 | | | | | | | |
| Moisture: | | | | | | | | |
| Heavy irri. ..... | 4.8 | 7.0 | 13.0 | 9.1 | 1.08 | 6.0 | 3.8 | 78 |
| Medium " ..... | 4.5 | 7.5 | 13.0 | 9.2 | 1.07 | 6.1 | 4.1 | 79 |
| Light " ..... | 2.9 | 6.7 | 11.8 | 8.3 | 1.06 | 6.0 | 4.1 | 77 |
| LSD 5% ..... | 1.1 | | | | | | | |
| 1946 | | | | | | | | |
| Open—heavy irri. ..... | 1.0 | 8.4 | 15.7 | 8.8 | 1.10 | 6.8 | 4.2 | 84 |
| Shade " " ..... | .4 | 7.9 | 15.6 | 8.3 | 1.14 | 6.4 | 4.0 | 82 |

per 100 gm of fresh stems and leaves) was reduced from 6.0 to 3.3 by the shades in the present field experiment and from 6.4 to 3.4 in the earlier sand-culture experiment. In connection with the previous experiment, it was stated (6) that the relative light intensities at midday in the summer and winter were respectively in the order of 10,000 and 1,000 fc. It was found on the basis of a calibration after publication that the photometer used for these light measurements was reading too high. Adjusted values of 7,000 and 700 would have been more nearly correct. In these earlier experiments, the pots both in the greenhouse and outdoors were well separated so that relatively more of the above-plant light reached the lower leaves than in the present field experiment.

It may be noted in the foregoing tabulation that the shades tended to increase plant height but to decrease the number of main stalk nodes. In other words, internode lengths were increased. The internode lengths of the longest fruiting branches were also increased; in the open the mean length of the fruiting-branch internodes was 8.3 cm and under the shades 11.2 cm. In both the present and earlier experiment there were fewer fruiting-branch nodes at low light than at high light; in the earlier experiment the number was reduced almost to half but in the present experiment only from 4.8 to 4.0.

*Boll and fiber properties:* The boll and fiber property responses to shade for 1947 are compared in table III with results obtained in similar experiment at the high moisture level during 1946. It will be recalled that the 1946 data are for weekly irrigations on both sides of each row which was the same schedule used in 1947 for the heavy irrigation treatment.

In both years—with heavy irrigation—there were reductions of varying magnitude under the shades in seed cotton per boll, in the seed and lint indexes and also in the fiber properties: tensile strength, weight per inch and percent of mature fibers. The increase in fiber length, like the decrease in fiber weight per inch, parallels the increased main-stalk and fruiting-branch internode lengths and the reductions in weight per unit length of internodes. The decrease in fiber strength with decreased carbohydrates under the shades (in this test with Acala p18) corresponds also with the within-variety findings developed in the previous paper (4) wherein treatments that increased carbohydrate levels increased fiber strength (for varieties other than the Acala p18).

Effects of Partial Defoliation: In this experiment, which was undertaken as a second means of reducing carbohydrates in cotton plants, a half of each old leaf and a half of each new leaf was cut away weekly starting at the time of first flowers. The leaves were not cut until they had attained a length of 6 or 8 cm, or upwards to a third or half of their ultimate length. The leaves were cut longitudinally with at least half of the mid-vein left intact. It may

TABLE IV

Effects of Removing One-Half of Each New Leaf Weekly From the Time of First Flowers on Carbohydrate Levels (Percent of Fresh Weight) and on Growth, Fruiting and Fiber Properties of Acala p18 Cotton at Shafter, Calif., in 1947

| Plant | Leal blades | | | Stems—middle third | | | Bolls—at 13 days | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Control* | *Half-leaf* | *LSD 5%* | *Control* | *Half-leaf* | *LSD 5%* | *Control* | *Half-leaf* | *LSD 5%* |
| ANALYSES | | | | | | | | | |
| Moisture | 76.5 | 77.2 | 0.5 | 73.0 | 74.3 | 2.4 | 86.2 | 86.7 | 0.8 |
| Hexose sugars | .09 | .08 | .05 | .48 | .25 | .18 | 1.91 | 1.71 | .23 |
| Sucrose | .30 | .27 | .07 | .82 | .50 | .29 | .03 | .03 | .02 |
| Starch | .42 | .51 | .07 | .73 | .49 | .44 | 1.26 | 1.09 | .26 |
| Sum | .81 | .86 | .12 | 2.08 | 1.20 | .83 | 3.20 | 2.83 | .40 |
| Total nitrogen | .78 | .77 | .09 | .22 | .22 | .05 | .28 | .29 | .02 |
| Sample wts each, gm | 1.07 | .48 | .08 | 25.0 | 14.6 | .85 | 2.31 | 2.14 | .22 |
| GROWTH AND FRUITING | | | | | | | | | |
| Height, cm | .... | .... | ... | 87.0 | 63.0 | 7.2 | .... | .... | ... |
| Nodes—main stalk, nos | .... | .... | ... | 22.9 | 21.8 | .7 | .... | .... | ... |
| Bolls per plant, nos | .... | .... | ... | 18.1 | 13.8 | 5.7 | .... | .... | ... |
| Leaves, plus stems, fresh, gm | .... | .... | ... | 412.0 | 289.0 | 76.5 | .... | .... | ... |
| Relative fruitfulness, bolls/100 gm | .... | .... | ... | 4.6 | 5.1 | 1.0 | .... | .... | ... |
| Yield seed cotton, relative | .... | .... | ... | 12.6 | 10.8 | .5 | .... | .... | ... |
| BOLL AND FIBER PROPERTIES | | | | | | | | | |
| Seed cotton per boll, gm | .... | .... | ... | 7.4 | 7.7 | 0.6 | .... | .... | ... |
| Seed index, gm/100 | .... | .... | ... | 12.0 | 12.0 | .4 | .... | .... | ... |
| Lint index, gm/100 | .... | .... | .. | 8.5 | 8.6 | .5 | .... | .... | ... |
| Fiber length—upper half, inches | .... | .... | ... | 1.05 | 1.05 | .03 | .... | .... | ... |
| " " mean, inches | .... | .... | ... | .78 | .80 | .03 | .... | .... | ... |
| " weight, μg | .... | .... | ... | 4.0 | 4.0 | .26 | .... | .... | ... |
| " strength, relative | .... | .... | ... | 6.3 | 6.6 | .26 | .... | .... | ... |
| " maturity, % | .... | .... | ... | 79.0 | 80.0 | 3.6 | .... | .... | ... |

be noted in table IV that the mean dry weights of the mature leaf blades as collected from the middle third of the main stalks for carbohydrate analyses were reduced from 1.07 to 0.48 gm. Since no special effort was made to cut away exactly half of each leaf, these relative leaf weights indicate only that after clipping, the remaining half of the leaves did not make extra growth to compensate for the part removed.

In the mature half-leaves from the middle third of the main stalks, there was a significant increase in starch concentration but the gain in sugar plus starch (6%) was not significant. Starch plus sugars in the middle third of the main stalks of the half-leaf plants was significantly lower by 42% than in the control stems; this loss was distributed almost equally between the hexose sugars, sucrose and starch. In the 13-day-old bolls there were small non-significant reductions in both hexose sugar and starch but the 11% reduction in the sum of sugars and starch fell short of significance.

Partial defoliation in terms of the means of 48 plants as measured on August 7 caused significant reductions in plant height (28%), number of main-stalk nodes (5%), fresh weight of leaves plus stems (30%) and the number of bolls per plant (23%). The yield of seed cotton as measured at the end of the season was reduced by 14%. The number of vegetative branches over 20 cm long was not changed by the half-leaf treatment. Measurements were not made of the number of nodes or lengths of fruiting branches. The relative fruitfulness of the half-leaf plants was higher (9%) than the controls—but this difference lacked significance.

The fact that there was a small gain, rather than a loss, in relative fruitfulness with partial defoliation would indicate that the reduction of stem carbohydrates from 2.04 to 1.20% did not increase shedding. This absence of a significant relation between carbohydrate levels in the stems and the relative fruitfulness of the plants is in accord with conclusions presented elsewhere (5). These conclusions were derived from a date-of-planting experiment conducted with both Acala and Stoneville cotton in adjacent plots during the same summer; the late planted cotton was retaining its bolls at the same time that the early cotton was shedding its bolls and the two plantings had similar levels of carbohydrates in their stems. Within-treatment scatter diagrams of plant weight against relative fruitfulness from several experiments including this one have shown a rather consistent tendency toward a lower relative fruitfulness of the larger plants.

The effects on boll and fiber properties of partial defoliation remain to be considered. As shown in table IV, there was surprisingly little effect of the half-leaf treatment on seed cotton per boll, seed and lint index (weight of 100 seed and lint on 100 seed), fiber weight per inch and per cent of mature fibers. The small differences found in these properties all lack significance but in each instance the differences tended to favor the half-leaf plants.

Fiber strength, in contrast with the lack of effect on the foregoing boll and fiber properties, was increased from 6.3 to 6.6 by partial defoliation; the difference was significant at the 5% level. This gain in fiber strength in association with reduced carbohydrate levels in the stems would place these results in line with the inverse relation found between varieties rather than in line with the direct relation that results from partial defruiting, drought and shading.

## Discussion

Growth Responses to Shading: Although there are no data on the light-intensity requirement for full photosynthetic activity in cotton leaves, inferences as regards ranges may be drawn from the findings of several investigators who have worked with other plants. Hoover, Johnson and Brackett (10), for example, found little increase in the apparent photosynthesis of young wheat plants above 1,000 fc at the $CO_2$ concentration of air when the intact tops were enclosed in tubes and illuminated laterally. Kramer and Decker (11) using intact seedlings found two species of oak to attain maximum $CO_2$ absorption at light intensities equivalent to one-third or less of full sunlight; loblolly pine showed maximum photosynthesis only with the equivalent of full sunlight. Verduin and Loomis (15) found segments of attached maize leaves to become light saturated at 2500 fc. These investigators reported ground intensities under full-grown and normally-spaced Iowa maize to be 30 to 40% of full exposure. On bright midsummer days it is evident that the lower leaves might be fully functional at midday but it would seem that this would not apply on cloudy days or during many of the morning and afternoon hours, nor could it apply well in August and September as the light intensities decrease. It may not be extraneous to recall, in this connection, the rainy and cloudy August of 1949 in Eastern Nebraska when unirrigated maize plants spaced 18 and 20 inches apart in 40-inch rows made better than average yields in response to the extra moisture; during the same season heavily fertilized and irrigated maize spaced 7 and 8 inches apart produced only 70% as much grain as they did during the previous year when August was bright and dry. The idea is extensively held that reduced light intensities caused by self-shading constitute one of the important factors limiting exceptionally high maize yields.

Thomas and Hill (14) working with alfalfa and wheat in glass-enclosed field-type plots show the hourly march of $CO_2$ absorption to rise more rapidly in the morning and to recede less rapidly in the afternoon than light intensities on a horizontal surface. The gains in $CO_2$ assimilation by alfalfa between 9 A.M. and noon (Sept. 15) amounted to only 10 to 15% whereas the light intensity between these hours increased approximately from 4800 fc to 7500 fc. From comparisons between bright and cloudy hours they deduced that there was no loss in apparent photosynthesis unless the light intensity dropped below 52% of the midday maximum; the data suggest light saturation at somewhat above 4,000 fc for this field alfalfa. This high light requirement, like the

findings of Heinecke and Childers (9), sets the behavior of field plantings, or trees, apart from the results commonly obtained with fully illuminated leaves or seedling plants.

In 1947 the ground intensities beneath cotton plants 110 cm tall that were spaced 12 inches apart in 38-inch rows were well below 5% of the intensities above the plants. At the half-height of the plants, the intensities were in the order of 25 to 30% of full light. On the basis of an average intensity of 6,000 fc for the midday maximum during August one-half of the leaves of the cotton plants would have received maximums as high or higher than 1800 fc for the brightest hour of the day. On the other hand, the leaves on the lower half of the plants would have operated, on the average, at intensities in the order of half or less of the 1800 fc during the best hours of the day; during the remaining hours the photosynthetic contribution of fully shaded leaves—those receiving only transmitted light—was probably nominal or almost negligible.

TABLE V

SUMS OF SUGARS AND STARCH IN COTTON LEAVES FROM THE MIDDLE THIRD OF THE MAIN STALK IN MID-JULY, THE LAST OF JULY AND MID-AUGUST UNDER WEEKLY IRRIGATION IN 1947 AT SHAFTER, CALIF. PERCENTAGES ON FRESH WEIGHT

| PLANTING[1] | MID-JULY | LAST JULY | MID-AUGUST |
|---|---|---|---|
| A—Acala p18, open ..... | 0.78 | 0.69 | 0.58 |
| B—Acala p18, shade .... | .53 | .70 | .41 |
| C—Acala p18 ..... | .96 | .97 | .68 |
| D—Stoneville 2B ..... | 1.09 | .87 | .78 |
| E—Acala p18, full-leaf .. | .97 | .82 | .64 |
| F—Acala p18, half-leaf .. | 1.08 | .87 | .64 |
| Means ..... | .90 | .82 | .62 |

[1] A and B are from the open and shade treatments of the present experiment. C and D are from the first planting of a date-of-planting experiment. E and F are from the half-leaf experiment. A and B were collected two days later than C, D, E and F.

LIGHT INTENSITY AND CARBOHYDRATES: From early July when the first flowers appear, and when there is little self-shading, the amount of self-shading increases through the first week in August as the plant expands; also, the maximum light intensities and day-lengths decrease. It becomes of interest for the foregoing reasons to examine the trends in carbohydrate levels in the leaves on the middle third of the main stalk through the sampling period. In considering these carbohydrate data and the variations from smooth trends it is pertinent to remember that each value represents a single sample with the variability associated both in the plants making up the samples and in the climatic conditions during and preceding the collections.

In addition to the data for carbohydrate levels in the leaves of the shade and half-leaf experiments, there are reported also in table V the results from the early planting of the date-of-planting experiment (5) which was conducted simultaneously in 1947. Allowing for exceptions provided by several of the samples, there were step-by-step reductions in carbohydrate concentrations in the leaves with the advance of the summer. In terms of the mean values, the concentrations of leaf sugars plus starch were only 69% as high in mid-August as in mid-July. During this interval there was a continued development of fruiting and vegetative branches; the control plants in the three tests increased in mean height from 63 to 107 cm (70%) and the dry weight of the middle third of the main stems increased from 32 to 79 gm (147%). The decrease in leaf carbohydrates in the leaves of the middle third of the main stem is indicated as being associated with the progression of self-shading as well as with the lower maximum light intensities and shorter days. It is reasonable to believe that in mid-August the heavily shaded leaves nearer the base of the plant had lower carbohydrates than those from the middle third of the main stems and, also, that fully exposed leaves near the top of the plant had higher concentrations.

During the same period, little consistent change has been found in the concentration of carbohydrate in the middle third of the main stems, but exceptions have been noted. With the greater leaf spread by mid-August, the total interception of light by entire plants would have increased, thus compensating perhaps for the lessened photosynthetic activity of shaded leaves. Beyond mid-August there is little further setting of bolls or vegetative expansion—in other words the carbohydrate requirements of the plants by that time are largely reduced to the development of previously set bolls and to respiration.

More general conclusions on light relations are possible from the data provided by the shade experiment. In terms of the plots receiving heavy irrigation some of the results were as follows:

| Measurement | Plants in shade as percentage of plants in open |
|---|---|
| Light above plants ..... | 32 |
| Carbohydrates in leaves ..... | 81 |
| Carbohydrates in stems ..... | 69 |
| Carbohydrates in young bolls ..... | 99 |
| Leaves plus stems, fresh weight ..... | 87 |
| Leaves, stems, bolls, fresh weight ..... | 66 |
| Bolls per plant, number ..... | 49 |
| Yield of seed cotton ..... | 53 |
| Relative fruitfulness ..... | 55 |

Assuming, on the basis of other data, a fresh weight of plants of 150 gm at the time the shades were installed (half the plants with first flowers), those in the open gained 686 gm in above-ground fresh weight between late June and August 21 and those under the shades 411 gm; of these gains, developing bolls represented 464 and 229 gm, respectively. Thus whether measured on August 21 in terms of weight of developing bolls (reduced to 50% in the shade), number of developing bolls (reduced to 49%) or weight of seed cotton in October (reduced to 53%)

the reduced light intensity was only about half as effective for reproduction as full light. On the other hand, during the period of shading to August 21, the total gain in fresh weight (plants plus bolls) was 60% as great under the shades as in the open. Assuming a linear relation (13, 14) between apparent photosynthesis and the percentage of normal maximum light, this 60% growth value at 32% of light would extrapolate to a requirement of 54% of full light for growth equal to that in the open. The 54% value can be regarded as little more than an indication but it agrees with the Thomas and Hill (14) value of 52% for field alfalfa. The reduction in fruit setting by the shaded plants merits consideration along rather different lines.

Relative Fruitfulness and Possible Auxin Relations: It seems doubtful that the poor setting of bolls by the shade plants should be attributed, at least directly, to the low carbohydrate levels associated with the low light intensities. Effects of the low light on growth regulators would seem to offer more promise as a means of accounting for the poor setting of bolls.

There has accumulated over past years what was regarded as excellent evidence in support of the nutritional theory of boll shedding, i.e., that new bolls are retained by the cotton plant only so long as the supply of sugars and protein constituents is ample for their continued development. Several experiments have lent themselves to this interpretation. In 1918 Ewing (8) found five varieties of cotton on a Mississippi hill soil to produce only one-third as much cotton as the same varieties growing on a valley soil and yet each group shed 55% of their bolls. Mason (12) found young bolls to shed promptly when Sea Island cotton was defoliated. When all bolls were removed from cotton plants in Arizona on July 26, 1926 (2) the plants increased their rate of vegetative growth and in a matter of 10 days the bolls removed had been replaced with a slightly larger number of new bolls; soon after this replacement of bolls the plants again started to shed young bolls more heavily. Another experiment showed that the systematic removal in 1927 of all flowers from alternate branches of cotton plants (unpublished) was without effect on the height of these plants or on the number of bolls they matured; the control plants produced an average of about two bolls on each of their longer fruiting branches, whereas there were four bolls on the corresponding even-numbered branches of the partially deflorated plants.

Our own more recent studies (5) have shown, on the other hand, that there is little or no difference in the carbohydrate and N levels in the stems of cotton plants between mid-July when there is little shedding and mid-August when shedding is heavy. Also, little difference was found in these stem levels or in those of young bolls in mid-August between old plants that were shedding heavily and a late planting that was not shedding. Girdling cotton plants (5) increased both carbohydrates and N but caused a great increase in boll shedding; N spray caused more rather than less shedding. These data pointed to the need of reconsidering the earlier experiments and the nutritional interpretation in search of some other explanation of boll shedding which would more nearly fit all of the facts.

By a recently proposed theory (5), the shedding of cotton bolls is controlled by the ratio of leaf mass to the number of developing bolls; environmental factors have been found to influence this ratio. On more theoretical grounds, it has seemed possible that shedding results when an anti-auxin or auxin-inhibiting material produced in developing cotton bolls reaches a high enough level in the fruiting branches to counterbalance the growth-promoting auxin produced by the plant. Recent data by others merit consideration in this connection. The results to be pointed to are from reports presented at the Seventh Cotton Defoliation Conference (January 14, 1953) and are on the basis of subsequent correspondence and approval by the investigators noted.

Using samples collected in the late summer, F. T. Addicott, V. B. Dawson and G. S. Sekera found a correlation between stages of leaf development and Avena coleoptile curvature; the auxin per leaf blade increased from the time of half-development to reach a prominent maximum just before the cotton leaves started to yellow. Associated with the increase and decline in auxin level there was evidence of a similar cycle in an auxin-inhibitor. H. R. Carns has found the ovaries of small cotton bolls (a few days old) to show no auxin transmission to agar blocks; when similar ovaries were placed on agar blocks containing 100 $\mu$g of IAA per liter, the IAA activity disappeared from the blocks. L. C. Brown reported, further, that spraying cotton plants with two-thirds gallon per acre of MH-30 (maleic hydrazide, now commonly regarded as an anti-auxin) caused upwards to 90% of bolls less than 25 days old to shed. The latter observation is in line with other data (7) showing increased boll shedding following the weekly spraying of the tops of plants with MH during the early flowering period. Spraying field plants weekly with 20 ppm beta-naphthoxyacetate has been observed previously (3) to increase significantly the number of bolls per plant and cause a slight increase in relative fruitfulness. On the other hand, the same concentration of the more active *p*-chlorophenoxyacetate which produced leaf symptoms resulted in larger plants but reduced both number of bolls per plant and relative fruitfulness.

The association of reduced fruitfulness with low light in terms of auxin relations would seem to accord with Murneek's results (13) with tomato plants. In winter greenhouses he obtained outstanding gains in numbers and weights of tomato fruits when the plants were sprayed—as a supplement to pollination—with beta-napthoxyacetic and *p*-chlorophenoxyacetic acids. But outdoors in the summer when the plants had good light exposures, his results were reported as being consistently negative.

It was pointed out in a previous paper that there are reasons for regarding the fruiting branches of the

cotton plant as being homologous to a raceme type of inflorescence. In cotton the internodes of the fruiting branches are elongated and, also, the leaves at the internodes reach full development rather than remaining repressed as bracts or scales. With the fruiting branch of cotton regarded as an inflorescence, it becomes more understandable why fruiting branches are absent on short-day cottons grown under long days and, also, why a day-neutral cotton grown without the benefit of temperatures above 80°F (1) should develop only large vegetative branches with fruiting branches absent or having only an internode or two. The effects of light intensities on the number of nodes of the fruiting branches—as differing from length-of-day and possibly temperature responses—appears to take the form of a degree of response rather than as one represented more nearly as a presence or absence.

On the basis of the foregoing discussions, the impression would result that cutting away a half of each leaf—as in the half-leaf experiment—should have reduced relative fruitfulness. The data of table IV show, however, that relative fruitfulness instead of being reduced was increased slightly—though not significantly—by the leaf clipping. Because of the possible significance of the half-leaf experiment, as a test of the boll number-leaf mass idea, the leaf blades and petioles-plus-stems of eight plants each from the control and from half-leaf treatments were weighed separately on August 7. The leaf blades constituted 41% of the weight of leaves-plus-stems in the control treatment and 39% in the half-leaf treatment; this difference was not significant. Scatter diagrams of relative fruitfulness against weight of leaves and stems under both treatments indicated a reduction in relative fruitfulness with increase in plant size that amounted to about one boll per 200-gram increase in weight of fresh leaves and stems. In view of this latter relation, it would seem that a slight but non-significant increase in relative fruitfulness of the half-leaf plants is in keeping with previous conclusions.

Boll and Fiber Properties: One is led to wonder at the remarkable stability in the properties of the seed and fibers produced by the cotton plant. Even under the influence of the somewhat drastic treatments represented by the heavy shading and the cutting-away of a half of each leaf in the present experiments, the changes affected in boll size, in the proportions of seed and lint, and in the length, strength and fineness of the fibers were minor. Similarly the effects of allowing only two bolls to develop on large plants, with the associated abundance of nutrients, have been found to be minor. Greater changes have resulted when the normal development of bolls has been prevented by drouth.

Without the protective mechanism of boll shedding, the cotton plant, due to deficiencies in organic nutrients, would undoubtedly produce smaller bolls, or bolls of diverse sizes with associated unfavorable ranges in the properties of the seed and fibers. Under such conditions the problems of cotton spinning would become far more complex and it seems doubtful that cotton could have come to occupy its present outstanding position as a textile fiber. The reproductive efficiency of the present-day cotton plant is already relatively high. Assuming then that one was able through the use of "hormone" materials of the right kinds and the right amounts, greatly to increase boll setting, would it not follow, inevitably, that boll sizes would be reduced and the walls of the fibers remain poorly thickened and the strength of the fibers greatly reduced? The fact, however, that adverse effects on the use-value of cotton is indicated as a probability with any great increase in the fruitfulness of the plant does not lessen the interest in trying to accomplish this as a means of furthering an understanding of boll shedding and of factors that influence the properties of cotton fibers. Studies of the effects on cotton yields from the use of "hormone" materials in areas where light intensities and temperatures are inclined to be low could, on the other hand, have a practical objective as well.

## Summary

The half-height light intensity among irrigated cotton plants 110 cm tall at Shafter, Calif., was found to be 30% as great and the ground intensity less than 5% as great as the intensity above the plants.

Reducing light intensities to 32% of full exposure by muslin shades erected at the start of flowering reduced the sum of sugar and starch in the leaves by 24%, in the main stems by 38% and in 14-day-old bolls by 8% (means of heavy, medium and light irrigation). The yield of seed cotton (heavy irrigation) was reduced by 47%, fresh weight of leaves plus stems by 13% and relative fruitfulness from 6.0 to 3.3. Plant height and lengths of longest fruiting branches were increased but the number of nodes decreased. There were small reductions under the shades in various boll and fiber properties other than fiber length—fiber length, like internode lengths, was increased.

Removing one-half of previous leaves and of each new leaf weekly from the start of flowering caused little change in the sums of sugar and starch in leaves or young bolls but there was a 42% reduction in the main stems. The half-leaf treatment reduced plant height by 28%, number of mainstalk nodes by 5%, fresh weight of leaves plus stems by 30% and yield by 14%; relative fruitfulness was increased slightly. Fiber strength was increased but there was little change in other boll and fiber properties.

The effects of the two treatments on fruitfulness are discussed in terms of ratios of vegetative weights to number of bolls (relative fruitfulness) and also, on the basis of recent literature, in terms of auxin and anti-auxin relations. The effects on fiber strength are considered in terms of carbohydrate relations.

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# RESPIRATION OF MAIZE GRAIN[1,2]

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Many data have been published upon the storage behavior of wheat (3, 12, 13, 14, 16, 19). Considerable work has been done on other seeds and grains (2, 4, 6, 15), but relatively little has been done on maize or corn. Bailey (1) found respiration of corn to be less affected by moisture than that of wheat with the materials he used. He observed also that freshly harvested corn had a lower rate of respiration than old corn rewet to the same moisture content. Semeniuk and Gilman (18) have reviewed the literature on storage of corn and the interaction of fungi in the deterioration of the product. Gilman and Barron (9) showed that fungi may be the major factor in heating of moist grain. It is probably significant that oats, with dead glumes on which saprophytic fungi can develop rapidly, heat more readily than wheat. Corn might be expected to be as resistant to mold as wheat.

The development of government storage of maize during recent years has emphasized the need of more knowledge of the behavior of this grain. The interaction of grain condition, weather factors and microorganisms in respiration and related processes within the storage bin are of particular significance in the successful storage of corn. Ventilation of the bin affects its content of $O_2$ and $CO_2$ as well as the temperature and humidity. All of these can affect respiration, growth of micro-organisms and undesirable enzymatic changes within stored grain.

The present study was undertaken to determine the effects of moisture content of grain, temperature, $O_2$ depletion accompanied by $CO_2$ accumulation, dormancy and the presence or absence of micro-organisms on the respiration of maize grain under controlled laboratory conditions. It is assumed that the data will be of value in storage problems as well as indicative of the physiological responses of this plant material.

## Materials and Methods

Most of the experiments were run with a single lot of hand-shelled, US-35 seed corn having an initial moisture content of 8%. This was moistened to the experimental percentages by adding the estimated quantity of water needed and holding at 4°C with frequent stirring for 3 days. The moistened grain was then equilibrated with air at room temperature and divided into moisture and experimental samples. Immature grain was taken directly from a field of hybrid corn for comparison with moistened, mature grain. The central part of each ear was shelled and used for two moisture and one respiration samples.

A special respiration flask, illustrated in figure 1, was used throughout. The lower neck of a 1,000-ml, wide-mouthed Erlenmeyer flask is restricted (*A*) so that a No. 8, two-hole rubber stopper (*B*) will fit securely into the recessed opening. Outlets from the flask consist of two Pyrex capillary tubes. The sam-

[1] Received March 26, 1953.
[2] Journal Paper No. J-2282 of the Iowa Agricultural Experiment Station, Project 1139. Tabular data and additional details of the work are included in a thesis, Respiration of Maize, by Hassan Ragai. Iowa State College Library, 1953.
[3] Present address: Ministry of Agriculture, Giza, Egypt.

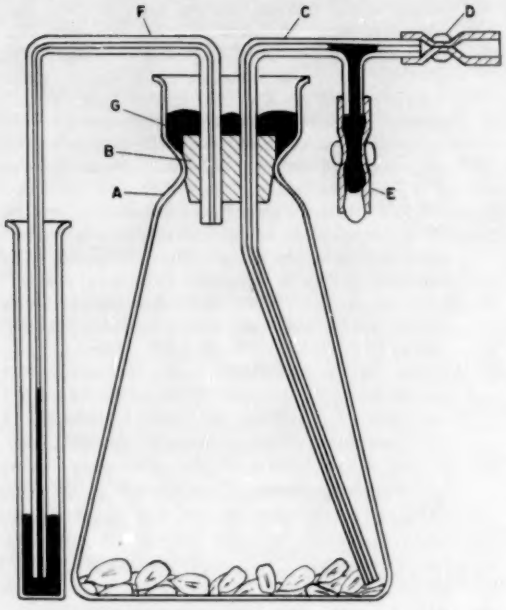


FIG. 1. An inexpensive, mercury-sealed respiration flask. See text for description.

pling tube (*C*) extends close to the bottom of the flask, and is closed at the outlet with a screw clamp on rubber tubing (*D*). About one inch from the outlet is a vertical arm in which mercury is held in a rubber tubing reservoir (*E*). A clamp on this reservoir can force mercury into the horizontal portion of the T and seal it against gas leakage. The other outlet (*F*) extends to a mercury seal in a mercury reservoir and serves as a manometer. When the sample is in place, the flask is stoppered and mercury (*G*) is poured into the neck of the flask to cover the rubber stopper. The advantage of the flask is that all outlets are sealed with mercury against the loss of carbon dioxide, and yet by lowering the mercury in the T-seal, samples of air may be taken readily. Glass-to-mercury seals are imperative in work of this type, since $CO_2$ may be lost rapidly through exposed rubber surfaces.

Flask volumes were obtained from the weight of water required to fill them, and sample volumes by displacement. Gas samples were withdrawn from the flasks at intervals through the tube *C* and analyzed in a Haldane gas analyzer. Total standard volumes of $O_2$ and $CO_2$ in the flasks were calculated from the free volume of the flask and the percentages of these gases in the sample. Calculations were corrected for gases withdrawn in sampling.

Production of $CO_2$ and of $O_2$ consumption were calculated for sampling intervals and for the entire experiment to the time of any particular sampling. Interval samples were used in estimations of the effects of $O_2$ depletion and $CO_2$ accumulation, and average rates to the sampling time at which accumulated $CO_2$ was nearest to 10% were used for the other comparisons. Respiration rates were calculated as ml $CO_2$ per kg dry weight of grain per day. Interval rates are designated $R$, and average rates to 10% $CO_2$ as $R_{10}$.

Ceresan-M was used, on the advice of Dr. C. S. Reddy, to disinfect certain seed lots for estimations of the effect of micro-organisms on respiration. One part of chemical was used in 10,000 parts of water and the grain soaked for 15 min. Germination was reduced an average of 5% by this treatment, while all molds and practically all bacteria were eliminated from this high quality, hand-shelled corn.

## EXPERIMENTAL RESULTS

Data were collected on the respiration of samples of maize grain in respiration flasks (cf. fig. 1). With the measurements made it was possible to separate out four major groups of data indicating: (a) the effect of moisture, (b) the effect of temperature, (c) the effect of $O_2$ depletion and $CO_2$ accumulation, and (d) the effect of micro-organisms upon the rate of respiration.

EFFECTS OF MOISTURE ON RESPIRATION: Artificially dried corn of seed corn grade, having an initial moisture content of 8%, was moistened to levels varying between 14 and 24% and its average respiration rate during the period when about half of the $O_2$ in the flask was being consumed was determined at three temperatures. Data are shown in figure 2. Respiration increased in an exponential manner with increas-

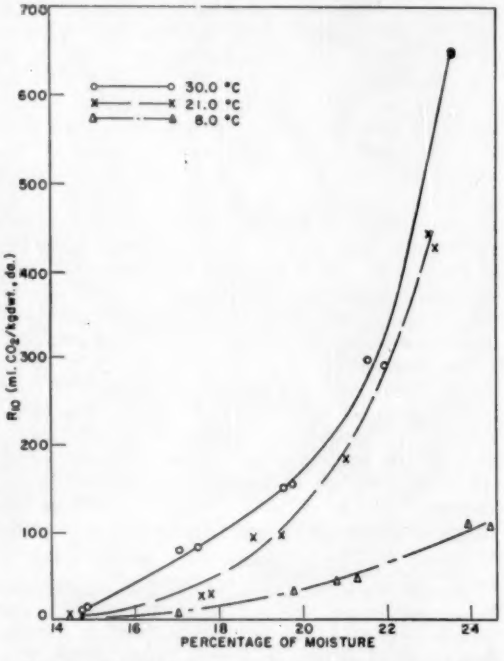


FIG. 2. Respiration of moistened, mature grain at three temperatures and varying moisture percentages.

ing moisture at all temperatures. The 21°C curve shows some irregularity, although it follows the same general pattern as the others.

Respiration was so slow at 8°C that the $CO_2$ in a 1000 ml flask containing 500 gm of corn at 14.9% moisture did not rise above 4% in 15 weeks, giving a respiration rate of less than 1 ml $CO_2$/kg dry grain per day. Such small quantities of $CO_2$ can be held only with a mercury-to-glass seal, such as was used here. Evidence that both seal and method were satisfactory is given by the fact that this reading fits a smoothed curve drawn through the respiration rates at the same temperature but with higher moisture percentages.

The rise in respiration approached or exceeded the doubling with each percentage increase in moisture content which has been reported by Bailey and Gurjar (3) as characteristic of wheat at moisture levels around 15%. In terms of corn storage it is significant that respiration of corn of 20% moisture at 8° was almost as low as that of 15% corn at 30°C, and respiration at 8° and 24% moisture was below that of 19% corn at 30°C.

The curves of figures 2 appear to rise exponentially, and we might assume that they would be described by the equation:

$$y = ae^{bx}, \qquad (1)$$

where $y = CO_2$ production rate; $a$ = intercept of the log curve; $e$ = base of natural logarithms; $x$ = moisture percentage, and $b$ = slope of the log curve. When the data are plotted as logs, we obtain the graphs of figure 3. The increase was relatively greater at lower moistures, with the breaks of the curves varying from 20% moisture at 8°C to 17% at 30°C. Two equations are required to describe these curves;

$$y_1 = a_1e^{b_1x}, \qquad (2)$$

at the lower moisture range and

$$y_2 = a_2e^{b_2x}, \qquad (3)$$

at the higher. The $y_1$ curves in figure 3 show approximately the same values of $b$ with different values of $a$ and $x$. The log rate of increase of respiration with moisture was thus not affected by temperature. The $y_2$ curve for 21°C appears to be high, and the $b$ values for the three temperatures more variable. It is doubtful, however, if these differences are significant in view of the small number of replications used.

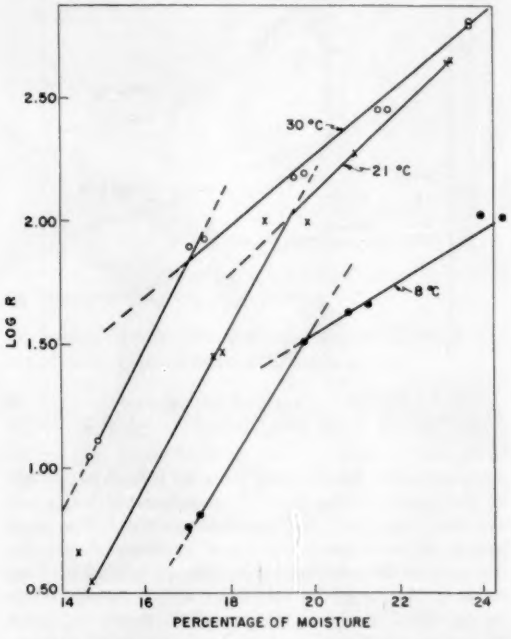


FIG. 3. A log plot of the data of figure 2. With increasing moisture, respiration followed the equation $y = ae^{bx}$, but two equations were required to cover the moisture range used. The differences of slope with temperature are not considered to be significant.

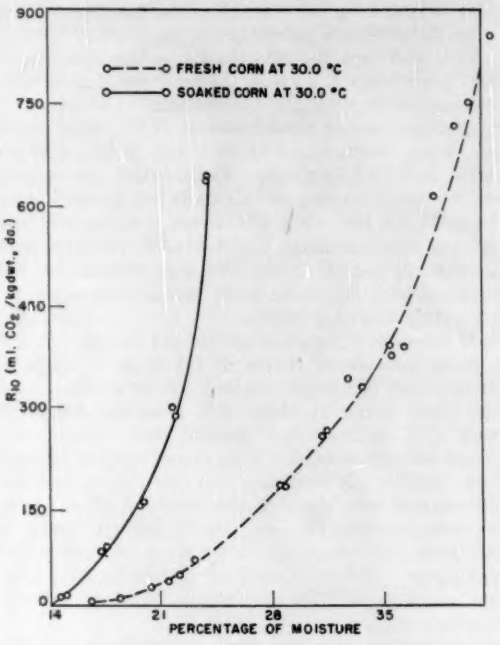


FIG. 4. Changes in the respiration of moistened, mature corn and immature corn with varying moisture content. The immature grain is considered to show the effect of inhibitors.

Rossman (17) found that immature grain was less injured by freezing than resoaked grain at the same moisture percentage, indicating basic differences in protoplasm structure. To determine whether immature and more or less dormant grain would respire at a lower rate than moistened, mature grain, ears were harvested as their moisture content in the field dropped from about 42 down to 16%, and respiration determinations were made with the shelled grain of individual ears. Respiration rates averaged for the

first 10% of $CO_2$ produced ($R_{10}$) are plotted in figure 4 against moisture percentage of the grain.

It is seen from the data that immature corn always had much lower $R_{10}$ values than moistened grain with corresponding moisture percentages. The order of magnitude was six times lower at 16.7%, eight times at 18.5%, seven times at 19.7 and 21.6%, and six times at 23.0% moisture. Germination percentages of the immature ears at 16.7 to 31.2% moisture were from 74 to 100, while the values for the still less mature ears containing 32.8 to 41.7% moisture were between 12 and 64. Even when high germination was obtained with immature grain, germination was slow, indicating dormancy effects.

If the data of figure 4 are plotted as logs, the immature corn shows curves of the type in figure 3, except that the slopes are less for both curves and the break comes at about 25% moisture, compared with 17% in moistened, mature corn. Bailey (1) found differences of this type extending into December. Neither his work nor ours provides a basis for determining how much of the observed effect is due to dormancy reactions, e.g., respiratory inhibitors, in the grain and how much to changing microbiological conditions. The uniformity of the results with both low and high moisture percentages, however, strongly indicates respiratory inhibition.

Effects of Temperature on Respiration: The data of figure 2 show both the effects of moisture and of temperatures on the respiration of corn. $Q_{10}$ values have been calculated for respiration of corn with comparable moisture percentages stored at different temperatures with the equation:

$$Q_{10} = \frac{\text{rate at t + 10°C}}{\text{rate at t°C}} \qquad (4)$$

For convenience, quotients for any temperature interval, $x$, are calculated from the relationship:

$$Q_{10} = (Q_x)^{10/x} \qquad (5)$$

The $Q_{10}$ values for the temperature interval 21 to 30°C were 3.82, 3.30, 1.58, 1.68 and 1.53 at moisture percentages of 14.7, 17.5, 19.6, 21.6 and 23.7. Values for the interval 8 to 21°C at the same moisture percentages were 3.61, 3.12, 2.40, 2.99 and 2.95, respectively. The averages and most of the individual $Q_{10}$ values are those expected of enzymatically controlled, chemical reactions, and they emphasize the importance of low temperature in reducing respiration rates in moist grain. A comparison of the effects of moisture and temperature indicates that a drop of 4°C gave approximately the same reduction in respiration as drying the grain to 1% lower moisture.

Effects of $CO_2$ and $O_2$ Concentrations on Respiration: With the method used, of sealing grain in flasks and measuring respiration by periodic analyses of the contained gases, respiration occurred in an atmosphere which was continuously decreasing in $O_2$ and increasing in $CO_2$ percentages. The individual $R$ values calculated for each sampling interval are therefore indications of the combined effects of these changes on the respiration of corn. Also, the values shortly before and after the time of the exhaustion of the $O_2$ content of the flask are indications of fermentation rates and possible Pasteur effects.

Decreasing respiration rates with $O_2$ depletion and varying moisture percentages are shown in figure 5. Germination percentages taken after the respiration run showed that there was no permanent injury of the grain, since all lots germinated 98 to 100%. The approximate point of $O_2$ exhaustion is marked on each of the curves. The presence of oil in the corn kernel led to RQ values below 1, and $O_2$ was exhausted faster than $CO_2$ was accumulated.

Since a low $R$ value at one reading led automatically to a high value at the next, some data points in the figure appear scattered. The general trend, however, is clear. Respiration rates dropped rapidly and exponentially with time in all experiments. Relative decreases were no less in the drier samples with their lower respiration rates, although a greater relative decrease might have been expected in the more active

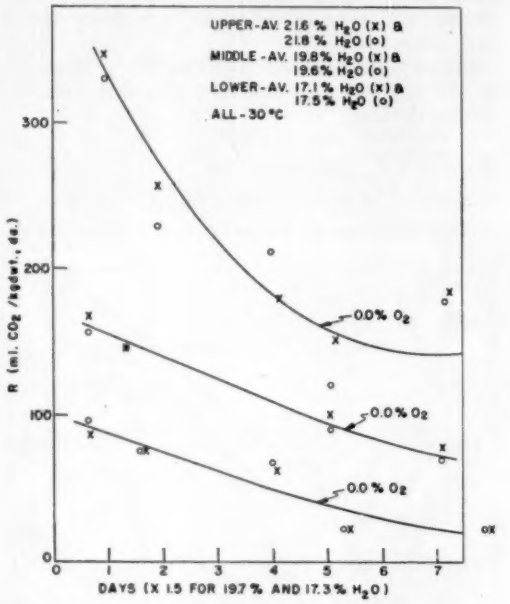
UPPER - AV. 21.6 % H₂O (x) &
21.8 % H₂O (o)
MIDDLE - AV. 19.8% H₂O (x) &
19.6% H₂O (o)
LOWER - AV. 17.1 % H₂O (x) &
17.5 % H₂O (o)
ALL - 30 °C
0.0% O₂
0.0% O₂
0.0 % O₂
R (ml. CO₂ /kg dr. day, dc.)
DAYS (X 1.5 FOR 19.7 % AND 17.3 % H₂O)

Fig. 5. Decrease of respiration of mature grain at three moisture levels with decreasing $O_2$ and increasing $CO_2$.

samples. And, finally, there was no consistent change in the rate curve as the $O_2$ was exhausted and all of the $CO_2$ was produced by fermentation. The data presented are a small fraction of those obtained. On the basis of the combined data the apparent high final value in the upper curve of figure 5 is considered to be an effect of the surging analysis shown by these flasks, in part because of poorly controlled temperature, and is not interpreted as a Pasteur effect since it was not commonly present. Compare, for example, the curves of figure 6.

As an average, the rate at zero per cent $O_2$ was

half of the initial values. It is not possible to separate the effects of $O_2$ depletion and $CO_2$ accumulation in these experiments, although, since the typical response was a continued reduction in rate of $CO_2$ accumulation after the $O_2$ was exhausted, it seems probable that both gases were affecting the rates.

Data on respiration of immature grain with decreasing $O_2$ and increasing $CO_2$ are graphed in figure 6. Results with this material were not markedly different from those with moistened corn except, as noted before, that the rates were lower. With immature corn at 30 and 20% moisture, respiration decreased about two thirds as $O_2$ was exhausted instead of about one half, suggesting more sensitivity to the effects of

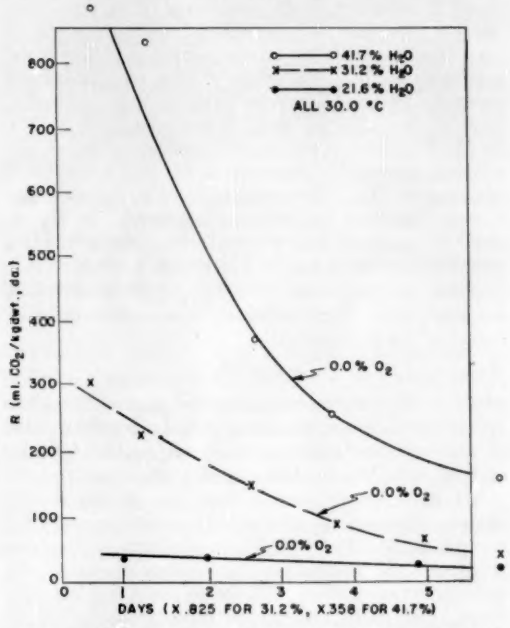


FIG. 6. Decrease of respiration of immature grain with $O_2$ depletion and $CO_2$ accumulation.

$O_2$ and $CO_2$. At the lowest moisture (21.6%) this trend may have been reversed.

EFFECTS OF MICRO-ORGANISMS ON RESPIRATION OF MAIZE: Micro-organisms, particularly molds (9, 13, 14, 18) play such a prominent part in the spoilage of grains that many workers have considered them to be the major factor in grain respiration. Milner *et al.* (14, p. 517) conclude: "The sharp increase in respiration of wheat at the so-called 'critical' moisture content is caused by the respiration of molds on and in the seed." These workers used various fungicides or fungistats mixed with the wheat rather than attempting complete sterilization. Non-toxicity was assumed where germination was not reduced by the treatment.

We obtained near sterilization of corn by soaking the grain for 15 minutes in a 1 to 10,000 solution of Ceresan-M. This treatment eliminated all molds,

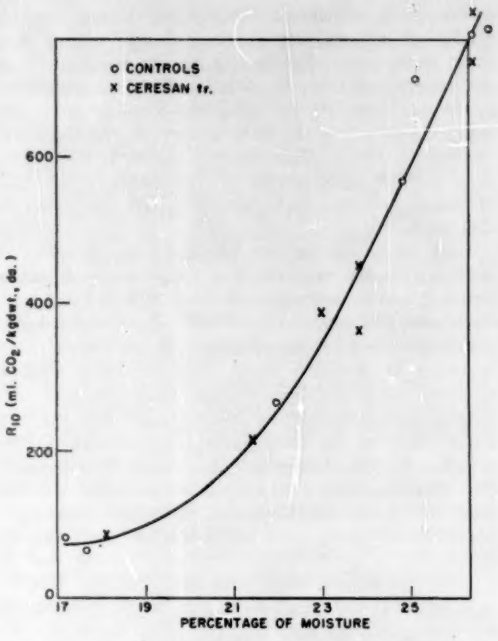


FIG. 7. Respiration of non-sterile (o) and near-sterile (x) grain of varying moisture content.

and many samples proved to be sterile when germinated on nutrient agar; others showed an occasional colony of a very slow growing bacterium, presumably a spore former. More severe treatments gave complete sterility but injured the corn and were discarded.

Data on the respiration of seven control samples of high grade corn and seven Ceresan-M treated samples are plotted in figure 7. Average germination at the end of the experiments was 91% for the controls and 86 for the treated samples. When the respiration rates of the non-sterile and near-sterile lots are plotted against moisture percentage, the data points give a satisfactory fit to a single curve (fig. 7), indicating no differences in respiration due to the elimination of all molds and most bacteria. Although the results might vary with other lots, and particularly with poorer grades of corn, they do indicate satisfactorily that maize grain has a respiration of its own which increases in an exponential manner with increasing moisture content.

## DISCUSSION

The respiration flask used in this work, and illustrated in figure 1, is an adaptation of a flask used by Kostychev (11). The glass-to-mercury seal is essential. Even ground glass joints may leak $CO_2$, and any exposed rubber can cause a large error when $CO_2$ concentrations on the two sides of the rubber are appreciably different. With this equipment it is possible to make many of the measurements commonly made with the Warburg apparatus at a small fraction of the apparatus cost. The use of macro-samples holds

errors to a satisfactory level, and makes possible studies of material not easily adaptable to the Warburg techniques. Errors due to the rapid drop in the respiratory rate, $R$, with $O_2$ depletion have been fairly well avoided by using the average $R$ at the sampling at which the $CO_2$ content of the flask was nearest to 10%. This average figure is designated $R_{10}$, and all comparisons of treatments, other than changing pressures of $O_2$ and $CO_2$, are compared on this basis.

Effects of Moisture: The data on the effect of moisture on the respiration of maize grain, shown in figure 2, are in general agreement with published results with other grains. At 21°C the respiration of maize increased 100 times from 4 ml kg/day at 14+% moisture to over 400 ml at 23%. Corn at 14% is considered safe for bulk storage while ear corn containing 23% moisture is too wet for safe cribbing under any but the more favorable conditions. Respiration, of course, is only a part of the storage problem. Generally more important is the ability of molds to grow rapidly at the higher moistures when temperatures are not so low as to inhibit their development.

Even the highest respiration rate observed here for mature corn, 650 ml at 30°C and 23.7% moisture, is not a particularly high rate for plant material. We have observed rates of more than 2000 ml/day for moistened corn at 45% moisture and of 5000 ml/day for green leaves.

The log plots of figure 3 indicate that the relative effects of moisture percentage were not affected by temperature, since the slopes of the lines are essentially identical. The significance of the double curve remains to be determined. Milner (14) and others have ascribed a rapid increase in the respiration of wheat at 16 to 18% moisture to the action of micro-organisms. The breaks in these log curves are at approximately the point at which more active mold growth might be expected, but the response is a lesser rate of relative increase rather than a greater. Also, the mercury-treated grain showed the same response as the non-sterile. Extrapolation of the curves of figure 3 toward the origin suggests that a third slope would be encountered at still lower moisture percentages, and scattered data with higher moisture percentages indicate more rate changes. It appears probable that a strictly logarithmic increase of respiration with moisture is limited to fairly narrow ranges. We might expect that these ranges would change with the condition of the grain and its previous treatment (15), particularly as these affect the microflora of the sample.

Log curves for immature grain showed a lower slope, corresponding to their lower respiration rate at comparable moisture levels. It is possibly a coincidence, but possibly significant, that the slope of the lower line for immature corn was identical with that of the upper line for mature corn.

Effects of Temperature: Data on the effect of temperature on respiration are shown in figure 2. As would be expected, respiration of maize grain increased with temperature and, except in the higher moisture levels of the 21–30°C comparison, showed $Q_{10}$ values around 3. Data of this sort give an equation for the effect of temperature on respiration of:

$$y = ae^{bt}, \qquad (6)$$

where $t$ is temperature in degrees C and the other values are as in equation-(1). A temperature coefficient of 3 means that the respiration of soft corn could be decreased on the order of 100 times by cooling the corn 75 or 80°F from the temperature of a warm fall day to near 0°F, and 15 times by cooling to freezing.

Effects of $O_2$ Depletion and $CO_2$ Accumulation: A reduction in the percentage of $O_2$ commonly reduces the rate of respiration, although there is considerable variability in the results of different experiments (5, 8). The effect of $CO_2$ accumulation is generally less, and is marked only at high concentrations (7, 10). Earlier work in this laboratory by Mr. C. M. Franco showed a slow response of maize respiration to reduced pressures of $O_2$ and a very slow response to $CO_2$. The results shown in figures 5 and 6 were therefore somewhat unexpected. If log $R$, data of figures 5 and 6, is plotted against time, a negative slope is obtained which shows a logarithmic decrease of respiration with $O_2$ depletion and $CO_2$ accumulation. The response is described satisfactorily by the equation:

$$y = ae^{-bx}, \qquad (7)$$

where $x = CO_2$ accumulation with time and the other values are those of equation-(1). The negative value of the exponent indicates that respiration ($y$) decreased with time in these closed containers.

The data are deficient in that they do not permit the separation of the effects of $O_2$ depletion and $CO_2$ accumulation. Some of the curves appear to show increased $CO_2$ production after $O_2$ was exhausted, the so-called Pasteur effect, but the response was not universal, and there are enough other irregularities in the data to suggest that these are chance variations.

Kidd (10) found that respiration rates decreased with increasing $CO_2$ concentration. From his experimental data he concluded: "The rate of anaerobic $CO_2$ production in plant tissues is depressed by carbon dioxide. Quantitatively the degree of depression at the temperature used appears to be proportional to the square root of the concentration of $CO_2$, over a range from 0 to 50% $CO_2$ at one atmospheric pressure. Above 50% the effect of increasing the concentration became gradually less marked. This depressant action of carbon dioxide is not limited to anaerobic $CO_2$ production, but occurs also in aerobic respiration in the presence of oxygen."

Effect of Micro-organisms: The pericarp of maize is normally invaded by micro-organisms, and complete sterilization without serious injury to the grain is very difficult. The treatment used here was effective in eliminating all mold and most bacterial growth with only a 5% reduction in germination of

the treated grain. When the respiration of treated and untreated grain was measured at varying moistures up to 27%, the two values were not significantly different. Such a result does not mean that the molds did not respire, but only that the total respiration of molds and grain did not rise above that of the grain alone under the conditions of our experiments. Much more work will be necessary to evaluate the relative roles of grains and micro-organisms in the respiration of the stored product. Reports that molds are responsible for most of this respiration (14) are probably due to the use of injurious chemicals as mold inhibitors. It is also possible that our treatment stimulated respiration of the treated grain above that of the grain alone in the grain-mold mixture. Tests of grain grown under sterile conditions, with and without specific inoculations, may be required to settle all points of this controversy. Our data, however, indicate that near-sterile maize is capable of high respiration rates, and that its respiration responds to moisture percentage changes in the same manner as non-sterile grain.

## Summary

The respiration of maize grain increased logarithmically with the percentage of added moisture according to the equation:

$$y = ae^{bx}.$$

The rate of increase, $b$, with moisture percentage, $x$, broke to a lower value at a moisture percentage which was lower with higher respiration intensities, so that two, and perhaps more, similar equations are required to cover the response of respiration to moisture.

Immature grain picked with a high moisture content showed the same moisture relations as moistened, mature grain, but rates were about one sixth as high at the same moisture percentages, presumably as an expression of respiration inhibitors in the partially dormant grain.

Respiration of maize increased with temperature, showing a $Q_{10}$ on the order of 3.

Respiration decreased in sealed containers as $O_2$ was depleted and $CO_2$ accumulated. The change was described by the equation:

$$y = ae^{-bx},$$

where $x = CO_2$ accumulation with time.

Respiration of a good grade of seed corn was not affected by a Ceresan-M treatment which eliminated all molds and most bacteria with negligible effect on the germination of the grain.

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# STUDIES IN PLANT METABOLISM. III. ABSORPTION, TRANSLOCATION AND METABOLISM OF RADIOACTIVE 2,4-D IN CORN AND WHEAT PLANTS[1, 2, 3]

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The use of 2,4-dichlorophenoxyacetic acid (2,4-D) as a selective herbicide has become quite extensive. In general, dicotyledonous plants are more sensitive to 2,4-D treatment than are most plants of the monocotyledonous types (7), although very little is known regarding the factors responsible for the difference in sensitivity. Recently, by using an isotopically labeled herbicide, Wood *et al.* (10) found that bean plants absorbed and translocated 2-iodo$^{131}$-3-nitrobenzoic acid (INBA) more readily than barley plants. Later, Mitchell *et al.* (8) extended their work to oat and corn plants. They suggested that the growth-inhibiting effect of INBA in the bean plant and its failure to produce significant inhibition in barley, oat and corn plants might be due to the differences in the manner in which INBA reacts with the plant constituents in each case.

Gallup and Gustafson (3) using 2,4-dichloro-5-iodo$^{131}$-phenoxyacetic acid were also able to demonstrate that the absorption of this growth regulator by the monocotyledonous plants is slower than by the dicotyledonous ones. The translocation to the apical portion of the plant is very rapid in broadleaved plants, but much less rapid in the grasses.

Holley, Boyle and Hand (4) using carboxyl-C$^{14}$-labeled 2,4-D demonstrated the presence of free 2,4-D in bean plant homogenate. Later Holley (5) using the same labeled preparation was able to show the presence of an ether-insoluble, water-soluble compound. Weintraub *et al.* (9), by using either carboxyl- or methylene-C$^{14}$-labeled 2,4-D, demonstrated that the C$^{14}$ from the applied 2,4-D in bean plants is incorporated into other substances within a few days. The radioactive carbon becomes distributed among a variety of plant constituents, including acids, sugar, dextrins, starch, pectin, protein and cell wall substances.

In a publication from this laboratory (2) it was found that with bean plants the radioactive 2,4-D was readily absorbed and the radioactivity was found in various parts of the bean plant. In a continuation of this study (6) two radioactive compounds, in addition to unchanged 2,4-D, were found in an 80% alcohol extract of the stems of bean plants treated with either $\alpha$-methylene- or carboxyl-C$^{14}$-labeled 2,4-D. It seemed worthwhile to extend this work to some 2,4-D resistant plants. The results here report the absorption, translocation and metabolism of radioactive 2,4-D by corn and wheat plants.

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[2] This work was supported by a grant from the Atomic Energy Commission.

[3] Approved for publication as Technical Paper No. 779 by the Director of the Oregon Agricultural Experiment Station. Contribution of the Department of Agricultural Chemistry.

## EXPERIMENTAL METHODS AND MATERIALS

The methods used for the combustion of the plant samples and the measurement of radioactivity have been described previously (2). A sample of carboxyl-C$^{14}$-labeled 2,4-D, with a specific activity of $5.3 \times 10^6$ counts per minute per mg, was synthesized in our laboratory and used in these experiments.

The corn plants (*Zea mays saccharata* var. Golden Bantam) and wheat plants (*Triticum vulgare* var. Hard Winter White) used in these experiments were grown under greenhouse conditions in potted soil. A 95% ethanol solution containing 0.1% radioactive 2,4-D and 0.5% Tween-20 (w/v) was used for treatment. The treatment was made to plants which were grown to the third or fourth leaf stage. Unless otherwise indicated, the corn plants received 20 $\mu$g of 2,4-D on the tip of the first or second leaf, while 10 $\mu$g were used in treating the wheat plants. In the study of absorption and translocation of C$^{14}$, the plants were harvested and sectioned into different parts as indicated in the tables. The sections were pooled and dried immediately in a vacuum oven at 60°C for 24 hours. An aliquot of each ground sample was analyzed for C$^{14}$ by the usual wet combustion procedure. The activity of $BaCO_3$ was counted and corrected to zero thickness. Duplicate determinations were made from all $BaCO_3$ samples. In every case an accuracy of 5% or better was realized.

For the identification and determination of 2,4-D and other radioactive compounds, the fresh plant material was homogenized in a Waring blendor with 80% ethanol. The materials were quantitatively transferred to a volumetric flask by rinsing several times with 80% alcohol and made to 250 ml with the same solvent. The radioactivity of the extracts was determined by direct plating in a stainless steel cup and then converted into radioactivity expressed as $BaCO_3$ by using a conversion factor (6). The radioactivity of the alcohol-insoluble residues was determined by the usual wet combustion procedure. The total activity of the plant was obtained from the summation of activity of the alcohol-soluble and alcohol-insoluble residues. The amount of 2,4-D present in the plant tissue was determined by the technique of one-dimensional paper partition chromatography. The extract, containing enough radioactivity for an accurate measurement, was applied to a spot about 7 cm from the end of strips of Whatman #1 filter paper (1 × 22 in) by means of a fine eye dropper. Usually duplicate strips were run. One was developed with a phenol-

TABLE I

ACCUMULATION OF RADIOACTIVITY IN CORN SEEDLINGS HARVESTED AT DIFFERENT TIMES AFTER TREATMENT WITH 20 μG CARBOXYL-$C^{14}$-LABELED 2,4-D

| PLANT PART | SPECIFIC ACTIVITY OF PLANT MATERIAL COUNTS/MIN/MG * | | | | AVERAGE DRY WEIGHT PER PLANT PART MG | | | | TRANSLOCATION AND ACCUMULATION OF $C^{14}$ % | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 DAY | 3 DAYS | 5 DAYS | 8 DAYS | 1 DAY | 3 DAYS | 5 DAYS | 8 DAYS | 1 DAY | 3 DAYS | 5 DAYS | 8 DAYS |
| 1st leaf † | 2.9 | 7.1 | ... | ... | 10.3 | 10.8 | ... | ... | 0.03 | 0.07 | ... | ... |
| 2nd leaf | ... | ... | 3.8 | 1.9 | ... | ... | 30.8 | 26.9 | ... | ... | 0.12 | 0.03 |
| 3rd leaf | 0.9 | 0.0 | 4.3 | 9.5 | 22.7 | 56.8 | 46.8 | 52.0 | 0.02 | 0.00 | 0.21 | 0.52 |
| 4th leaf | 3.7 | 3.7 | 28.0 | 21.0 | 5.7 | 48.8 | 23.5 | 60.2 | 0.02 | 0.17 | 0.68 | 1.33 |
| 5th leaf | ... | 14.1 | ... | 14.3 | ... | 9.7 | ... | 16.9 | ... | 0.13 | ... | 0.25 |
| Stem & sheath | 90.7 | 33.1 | 121.3 | 86.0 | 35.7 | 74.1 | 50.5 | 43.2 | 3.06 | 2.33 | 6.26 | 3.90 |
| Root | 9.2 | 4.1 | 10.4 | 15.0 | 126.5 | 148.5 | 160.5 | 131.6 | 1.10 | 0.58 | 1.73 | 1.83 |
| Total | ... | ... | ... | ... | ... | ... | ... | ... | 4.23 | 3.28 | 9.00 | 7.86 |

The plants harvested on the first and third days were treated on the second leaves and the plants harvested on the fifth and eighth days were treated on the first leaves. The treated leaves were discarded. Each group consisted of 10 plants which were grown to the fourth-leaf stage. (1 μg 2,4-D = 5370 counts per minute.)

* Counts/min/1 mg dry sample.

† Lowermost leaf.

$H_2O$ and the other with an ammonium hydroxide-*n*-butanol (1:4) system. After drying, the chromatogram was cut into 1-cm sections starting from the original spot and the radioactivity of each section was determined directly. A Tracerlab windowless gas flow counter was used when the radioactivity of the chromatogram was low. The following expressions were used for the determination of relative radioactivity of compounds containing $C^{14}$ in 80% alcohol extract and in calculation of the amount of 2,4-D present in fresh plant tissue:

$$\frac{\text{Radioactivity of the spot}}{\Sigma \text{ radioactivity of each section}} \times 100 = \text{Relative radioactivity in per cent}$$

$$\frac{\text{Total alcohol-soluble activity} \times \text{relative activity}}{\text{Specific activity of 2,4-D} \times \text{weight of fresh sample in gm}} = \text{Amount of 2,4-D per gm fresh sample}$$

## RESULTS AND DISCUSSION

In all experiments with corn and wheat seedlings, the accumulation of $C^{14}$ activity in the nontreated parts of the plants was much lower than was found in bean plants (2) as indicated in tables I and II. Corn plants translocated and accumulated slightly more $C^{14}$ than did the wheat seedlings. When 2,4-D was applied on the tips of the lowermost leaves of corn plants, the absorption and translocation of $C^{14}$ was found to be somewhat higher than when 2,4-D treatment was made on the second lowermost leaves. Most of the activity accumulated in the root, stem and sheath of the treated corn plants. The growing leaves (4th or 5th) usually showed a higher specific activity than was found in the other leaves, which indicated that the transport of $C^{14}$ to the apical portion of the plant is more rapid. In a separate experiment, which is not included in table I, the results indicated that when 2,4-D was applied to the first leaf the sheath of the first leaf had a specific activity of 447 cpm per mg dry sample while the sheath of the second leaf contained 8.7 cpm per mg, and of the third leaf 9.4 cpm per mg. The great difference in $C^{14}$ activity between the sheaths may be due to impedients in them or in the stems which interfere with the transport of 2,4-D

TABLE II

ACCUMULATION OF RADIOACTIVITY IN PARTS OF WHEAT SEEDLINGS WHICH WERE HARVESTED AT DIFFERENT TIMES AFTER TREATMENT WITH 10 μG CARBOXYL-$C^{14}$-LABELED 2,4-DICHLOROPHENOXYACETIC ACID TO THE 1ST LEAF

| PLANT PARTS | SPECIFIC ACTIVITY OF PLANT MATERIAL COUNTS/MIN/MG * | | AVERAGE DRY WEIGHT PER PLANT PART MG | | TRANSLOCATION AND ACCUMULATION OF $C^{14}$ % | |
|---|---|---|---|---|---|---|
| | 4 DAYS | 7 DAYS | 4 DAYS | 7 DAYS | 4 DAYS | 7 DAYS |
| 2nd & 3rd leaves † | 31.3 | 58.1 | 20.1 | 5.7 | 1.31 | 0.69 |
| Stem & sheath | 23.6 | 11.2 | 12.6 | 9.9 | 0.62 | 0.23 |
| Root | 5.8 | 3.2 | 9.1 | 13.5 | 0.11 | 0.09 |
| Total | ... | ... | ... | ... | 2.04 | 1.01 |

Each group consisted of 20 plants. Plants were grown to third-leaf stage. (1 μg 2,4-D = 5370 counts per minute.)

* Counts/min/1 mg dry plant sample.

† 2nd & 3rd lowermost leaves.

or radioactive compounds from the treated leaf to the nontreated one.

In the experiment with wheat seedlings, only between 1 to 2% of applied $C^{14}$ activity was found in plant parts from which the treated leaf was excluded. Most of the activity was present in the growing leaves, less in the stem and sheath, and least in the root. This pattern differed slightly from that found in the experiment with corn plants.

A fairly high accumulation of radioactivity in the untreated part of the treated leaf in the corn plant, as indicated in table III, revealed that 2,4-D was also readily absorbed by the resistant plant. This free 2,4-D may be found in a relatively large concentration in the treated leaf of the plant harvested after one day of treatment. It was comparable to the concentration which was found in bean stems (6) yet no physiological effect was observed in the corn plant. The uneven distribution of 2,4-D and also the difference in relative concentration found in the alcohol extract of plant parts in both one-day and three-day samples suggested first, that the transport of 2,4-D toward apical parts (4th leaf) is quicker than its movement into the root; and second, 2,4-D may be incorporated into other compounds more rapidly in the root than in the stem. The 2,4-D concentration dropped considerably in all plant parts of samples harvested three days after treatment, while at the same time, the $C^{14}$ activity in other alcohol-soluble compounds and the alcohol-insoluble residue was increased.

The results of the determination of free 2,4-D and other 80% alcohol-soluble compounds containing $C^{14}$ in the 2,4-D treated leaf (the treated section was excluded) of both corn and wheat plants are shown in figures 1 and 2. The corn plants were harvested in groups of four at 1, 3, 7, 9 and 14 days and the wheat plants were harvested in groups of ten at 1, 4 and 7 days after treatment. Only the treated leaves were used in this study. Pure radioactive 2,4-D was found to have an $R_f$ value between 0.72–0.76 in the phenol-$H_2O$ system and 0.62–0.64 when butanol-ammonium hydroxide (4:1) was used. A radioactive spot with an identical $R_f$ value developed with either solvent was found in the alcohol extract of the corn and wheat plants. The radioactive spot was eluted with alcohol and was subsequently co-chromatographed with a known amount of 2,4-D in both solvents. In each chromatogram, only one spot corresponding to the 2,4-D was found. Further evidence of the identity of the 2,4-D was given when a known amount of 2,4-D was added to the plant extract. The subsequent determination of the radioactivity of the paper chromatogram gave an increase in the activity of the 2,4-D spot which corresponded to the amount of 2,4-D added.

It is evident that the 2,4-D becomes a component of several compounds. The compound in the corn and wheat leaves which is quantitatively most important is one which we have designated as unknown 3, with an $R_f$ value of 0.83 in phenol-$H_2O$ and 0.85 in butanol-ammonium hydroxide. The amount of this compound increased with time for nine days to 58% and then remained relatively constant in the corn plants. Concomitantly the level of 2,4-D decreased. A significant but much smaller amount of the compound designated as unknown 1 was present. This compound has an $R_f$ value 0.57 in phenol-$H_2O$ and 0.04 in butanol-ammonium hydroxide and was the major radioactive alcohol-soluble compound formed in bean plants (6). In the time course study with wheat seedlings, a maxi-

TABLE III

DISTRIBUTION OF RADIOACTIVITY IN CORN PLANTS AFTER TREATMENT WITH 20 μG CARBOXYL-$C^{14}$-LABELED 2,4-D TO EACH PLANT. 2,4-D WAS APPLIED ON THE TIPS OF THE 2ND LEAVES

| | TOTAL RADIOACTIVITY IN PLANT PARTS | 80% ALCOHOL SOLUBLE | | 80% ALCOHOL INSOLUBLE | | $C^{14}$ FROM 2,4-D IN ALCOHOL EXTRACT OF PLANT PARTS † | 2,4-D PER 1 GM FRESH SAMPLE |
|---|---|---|---|---|---|---|---|
| | | TOTAL RADIOACTIVITY | SPECIFIC ACTIVITY * | TOTAL RADIOACTIVITY | SPECIFIC ACTIVITY * | | |
| | $cpm \times 10^3$ | $cpm \times 10^3$ | $cpm \times 10^3/gm$ | $cpm \times 10^3$ | $cpm \times 10^3/gm$ | % | μg |
| Expt. 1. Harvested after one day of treatment, 20 plants in this group | | | | | | | |
| Untreated part of treated leaf, 4.2 gm | 240.2 | 236.0 | 56.0 | 4.2 | 1.00 | 69 | 7.21 |
| 2nd leaf sheath, 3.8 gm | 81.7 | 80.5 | 21.2 | 1.2 | 0.32 | 57 | 2.25 |
| 4th leaf, 6.3 gm | 17.3 | 16.5 | 2.6 | 0.75 | 0.12 | 90 | 0.43 |
| Stem, 2.1 gm | 20.4 | 19.9 | 9.6 | 0.54 | 0.26 | 82 | 1.46 |
| Root, 27.8 gm | 40.1 | 36.1 | 1.3 | 4.0 | 0.14 | 56 | 0.14 |
| Expt. 2. Harvested after three days of treatment, 18 plants in this group | | | | | | | |
| Untreated part of treated leaf, 4.6 gm | 111.0 | 104.0 | 22.6 | 7.0 | 1.50 | 13 | 0.55 |
| Stem & sheaths, 13.4 gm | 44.2 | 27.7 | 2.1 | 16.5 | 1.10 | 56 | 0.22 |
| Root, 26.8 gm | 11.0 | 7.0 | 0.26 | 4.0 | 0.15 | 10 | 0.005 |

(1 μg 2,4-D = 5370 counts per minute.)
* Counts per minute per 1 gm fresh sample.
† Data obtained from paper chromatograms.

mum formation of unknown 3 was observed after 24 hours of treatment. Both unknown 1 and unknown 2 (6) were also present.

In an earlier report from this laboratory (6) it was found that the unknown 1 formed in bean plants is a 2,4-D complex since treatment with dilute acid or emulsin released free 2,4-D. Unknown 3, when treated in a like manner, also gave free 2,4-D. Therefore, it is tentatively suggested that this complex may be similar in nature to unknown 1, but that it may involve conjugation of 2,4-D with different compounds. The formation of the complex as was suggested earlier might be a detoxification mechanism in bean plants. The formation of unknown 3 might be a similar mechanism used by the corn and wheat seedlings. At any rate, there is a marked difference in the type of compounds formed after treating the bean plant (susceptible) on one hand and the corn and wheat plants (resistant) on the other.

At the present date, no information is available regarding the identity of all of these unknown compounds. However, in preliminary work, a small but isotopically pure sample of residue-$C^{14}$-labeled unknown 1 and unknown 3 had been isolated and hydrolyzed. The unknown 1 was hydrolyzed by boiling with 2 *N* HCl for 2 hours while with unknown 3, 2 *N* $Ba(OH)_2$ was used. After the solutions were neutralized, the paper chromatograms of these hydrolysates were developed with phenol-$H_2O$ or butanol-ammonium hydroxide. Many radioactive spots found on these chromatograms revealed the complexity of both unknown 1 and unknown 3 molecules. (Radioactive spots found in chromatograms from residue-$C^{14}$-labeled unknown 1 developed in phenol-$H_2O$ were at $R_f$ 0.00, 0.21, 0.28, 0.35, 0.42, 0.55 (unk. 1), 0.68, 0.74 and 0.84; radioactive spots found in chromatograms from residue-$C^{14}$-labeled unknown 3 developed in butanol-ammonium hydroxide were at $R_f$ 0.03, 0.11, 0.34, 0.48, 0.62, 0.70, 0.85 (unk. 3) and 0.99.) The residue-$C^{14}$-labeled unknown 3 was prepared from corn seedlings which were exposed to $C^{14}O_2$ for 3 days under greenhouse conditions and then treated with non-radioactive 2,4-D. The treated leaves were harvested after 24 hours and homogenized with 80% alcohol. Paper chromatograms of this alcohol extract were prepared and the radioactive spot corresponding to unknown 3 was cut off and eluted with alcohol. The eluate was redeveloped with a different solvent system. Three different solvent systems were used, namely, phenol saturated with water, ammonium hydroxide and *n*-butanol (1 : 4) and *n*-butanol-propionic acid-$H_2O$ (12 : 5.6 : 8). This process was repeated until only one radioactive spot appeared. The residue-$C^{14}$-labeled unknown 1 was prepared from bean plants in the same manner.

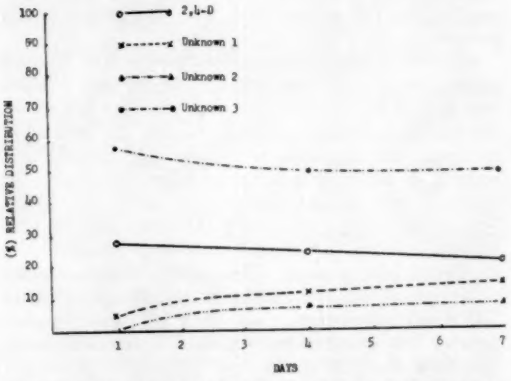


FIG. 1. Per cent distribution of 80%-alcohol-soluble radioactive compounds present in the leaf of corn seedlings treated with 20 μg of carboxyl-$C^{14}$-labeled 2,4-D. Plants harvested after varying intervals of time.

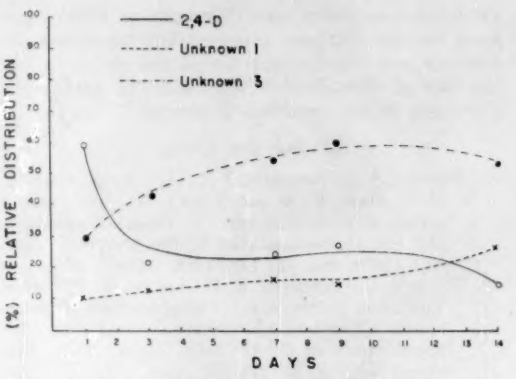


FIG. 2. Per cent distribution of 80%-alcohol-soluble radioactive compounds present in the leaf of wheat seedlings treated with 10 μg of carboxyl-$C^{14}$-labeled 2,4-D Plants harvested after varying intervals of time.

Further work regarding the identity of unknown 1 and unknown 3 is in progress and will be reported in a separate paper.

## SUMMARY

A study has been made of the absorption and translocation of carboxyl-$C^{14}$-labeled 2,4-D by the corn and wheat plants. It was found that this growth regulator is absorbed by the monocots but at a slower rate in comparison with bean plants. The translocation of $C^{14}$ to the apical portion of the corn and wheat plants was very slow. There seems to be a block in the intercalary meristem of the monocotyledonous stems and leaves of young plants.

At least a part of the applied 2,4-D is incorporated in a few days into other compounds. Two major compounds which we designated as unknown 1 and unknown 3 were found in the 80% alcohol extracts of leaves from both 2,4-D treated corn and wheat plants. The latter unknown, which was found in a greater concentration, indicated a difference in metabolism of 2,4-D from that found in the bean plants.

The identity of the major unknown radioactive compounds has not been established. However, the complexity of the molecule of these two 2,4-D conjugates has been demonstrated.

The question of how the monocotyledonous and

dicotyledonous plants react differently on 2,4-D treatment has not yet been answered but the slower absorption and translocation within the monocots and the rate of detoxification (formation of 2,4-D complex) may be the contributing factors.

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# INHIBITION OF GROWTH OF CHLORELLA PYRENOIDOSA BY BETA-EMITTING RADIOISOTOPES[1, 2]


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Many studies have been made during the past 50 years of the effects of ionizing radiations on plants, animals and microorganisms, but to our knowledge no extensive studies have been made on the effect of these radiations on Chlorella. Neither have attempts been made to grow this organism in the presence of radioactive elements, except in tracer experiments. Mutations of Chlorella have been produced, however, by Granick (3) with X-ray.

This paper reports (1) the methods used in growing algae in the presence of beta-emitting radioactive elements, and (2) the effect of several concentrations of tritium oxide, phosphorus-32, sulphur-35 and the equilibrium mixture $Sr^{90}$-$Y^{90}$ on the growth of Chlorella. The results presented, which are largely descriptive, are preliminary to other extensive studies now being made on the effect of tritium oxide on cellular processes and biosynthetic reactions of Chlorella.

## Materials and Methods

The culture of Chlorella, #7516 of the American Type Culture Collection, was grown on agar slants and transfers were made at monthly intervals. Algae to serve as inocula in experiments were transferred from slants at approximately 6-week intervals to one liter of sterile inorganic nutrient solution (Knop, modified). A modified, low-form, 3-liter culture flask mounted on an electrically driven rocker platform served as the container. The algae were grown under white fluorescent light of 100 to 300 fc, and aerated with a stream of 8 to 9% $CO_2$ in air. After 4 to 6 days' growth 900 ml of solution were drawn off 2 to 3 times a week and replaced with sterile nutrient solution. The algae withdrawn served as a source of inoculum for the experiments with the beta-emitting isotopes.

Algae were grown in the presence of each of the four isotopes in 20 × 150 mm tubes. The nutrient solution was that of Myers (4). Nutrient solutions containing tritium oxide were prepared as follows. Fifteen ml of nutrient solution, adjusted to pH 5.5, and distilled water were added to each tube. After autoclaving the solutions were cooled and tritium oxide was added. Cells to serve as inocula were removed from the continuous culture, centrifuged and then suspended in sterile nutrient solution. The suspension was adjusted to an optical density of 0.60 (660 mμ, Beckman Model DU Spectrophotometer) and then 1.0 ml of the suspension was added to each tube. The final volume in each tube was 18.0 ml.

Nutrient solutions containing the other beta-emitting isotopes were prepared somewhat differently. Six ml of nutrient solution were added to each tube. After sterilization 0.01 to 0.02 ml of 0.1% methyl red in alcohol was added. The radioactive isotope was added,


[1] Received March 30, 1953.

[2] This paper is based on work performed under contract No. W-31-109-Eng-52 for the U. S. Atomic Energy Commission.

and the solution was adjusted to pH 5.5 to 6.5, as judged by the indicator color, with KOH. If the pH of the solution dropped below 5.5 on aeration additional KOH was added. Sterile water and the inoculum were added. The final volume was usually 11.0 ml.

Carrier or acid, or both, were present in stock solutions of all radioisotopes, except tritium oxide. The pH of the solution of this isotope was approximately 7.0. The carrier and acid prevented excessive deposition of radioactivity on the walls of containers. Concentrations of phosphorus-32, expressed as $\mu$c/ml of final solution were those at the start of an experiment.[3] Concentrations of other isotopes did not change significantly during the experimental period.

During the growth of the algae, in a continuously exhausted hood, the light intensity (white fluorescent) incident on the tubes was approximately 400 fc, and the gas bubbled through the tubes was an 8 to 9% $CO_2$ in air mixture. It flowed at the rate of approximately 100 ml per minute. The temperature of the nutrient solution was kept at 25 to 27°C.

Growth of the algae was followed over a three-day period by making daily light absorption readings[4] at 660 m$\mu$. Controls, identical to experimental tubes except for radioactivity, generally showed logarithmic growth and a generation time of approximately 12 hours. Separate experiments showed a linear relationship between light absorption and cell mass (but not cell numbers) within the range in light absorption reported.

## Results

The growth of algal cultures, in log $I_0/I$ values, was plotted against time. Figure 1 shows the changes in light absorption by algae growing in the presence of 5 to 40 mc/ml $H^3_2O$ (1600 to 13,000 rep. (6) per day, calculated). Each point represents the average of the light absorption of duplicate tubes in a single experiment. Similar results have been obtained in 3 additional experiments with $H^3_2O$. It is evident from figure 1 that cell growth was not appreciably inhibited during the first day, but thereafter inhibition was a function of the dose of radiation. At the highest level of radiation growth was inhibited markedly at 72 hours.

Algae grown in the presence of other isotopes were exposed to the following dose rates of radiation (in rep. per day (calculated); $P^{32}$, 1000 to 5900; $Sr^{90}$-$Y^{90}$, 800 to 3200; $S^{35}$, 330 to 2700). Figure 2 shows an example of the changes in light absorption by algae growing in the presence of $P^{32}$. It will be noted that the weight of cells in the solution containing the highest level of $P^{32}$ was less at 72 hours than that in the control solution. It will also be noted that growth in this experiment was more erratic than that shown in figure 1. Presumably this was due to the lower levels of radiation used and to our inability, because of the method used, to control the nutrient composition as precisely as in the experiments with tritium oxide.

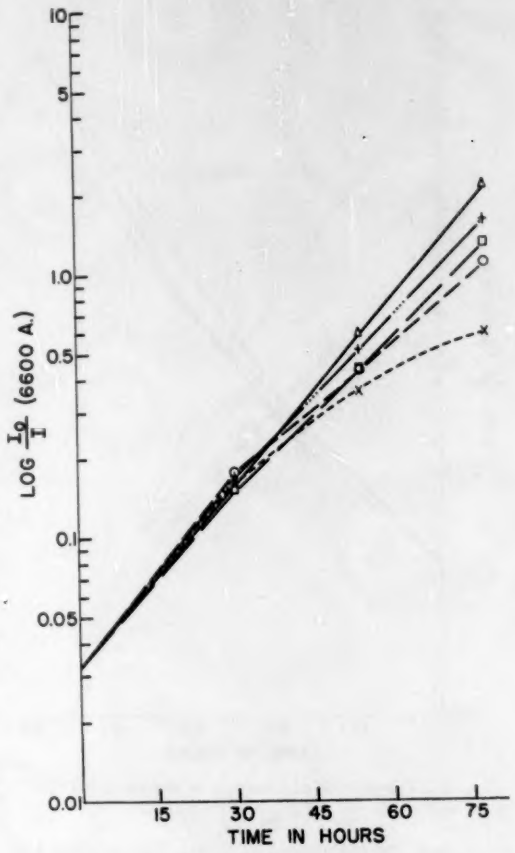


Fig. 1. The growth of Chlorella in the presence of tritium oxide. △—Control, +—5 mc/ml, □—10 mc/ml, ○—20 mc/ml, ×—40 mc/ml.

The growth capacity of algae following exposure to $H^3_2O$, $P^{32}$, $Sr^{90}$-$Y^{90}$ or $S^{35}$ was tested by subculture in non-radioactive nutrient solution. Algae grown for 3 days in radioactive nutrient solution were allowed to stand[5] an additional 3 days in the refrigerator in the

[3] All radioisotopes used in these studies were calibrated or recalibrated, by personnel of the Section's Analytical Group.

[4] Readings at 24 and 48 hours were made directly on the tubes, after removal of the aerators, in which the algae were growing. Readings at 72 hours were made on diluted samples if the log value was above 1.5. Loss of volume from the tubes during the growth period was minimized, except in those experiments with $H^3_2O$, by bubbling the air-$CO_2$ mixture through water before passage into the growing cultures.

[5] In other experiments algae were centrifuged directly from the radioactive solution and then taken up in fresh nutrient solution. These cells and duplicates not centrifuged from the radioactive solution were stored for 3 days in the refrigerator. Then each lot of cells, free of radioactivity, except that incorporated in the cells, was grown in non-radioactive nutrient solution. Growth after 3 days subculture of those cells stored 3 days in the presence of radioactivity was approximately 75% of that of the cells centrifuged immediately after the growth period ended.

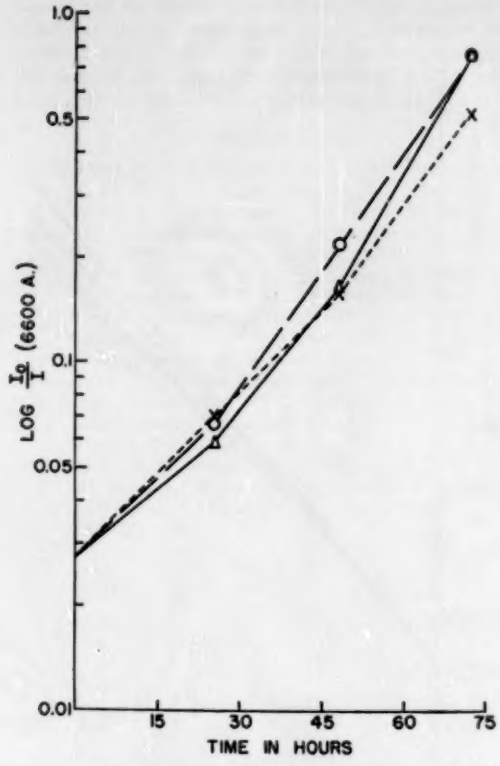


FIG. 2. The growth of Chlorella in the presence of $P^{32}$. △—Control, ○—77 μc/ml, ×—155 μc/ml.

presence of the radioactive solution. The solution was then decanted from the cells, the cells resuspended in fresh nutrient solution and then centrifuged (2 minutes at approximately 10,000 rpm). The supernatant solution was discarded and the cells resuspended in fresh nutrient solution. All suspensions were adjusted to the same log $I_o/I$ value (660 mμ) and then used as inocula for non-radioactive nutrient solutions. Growth of the cells was followed for 3 days.

Cells exposed to tritium oxide (fig. 1), when subcultured on inactive media gave the results shown in figure 3. Cells, exposed to $P^{32}$ (fig. 2), when subcultured on inactive media gave the results shown in figure 4. The growth of experimental and control cells shown in these figures varied as a function of the concentration of isotope in which the cells were originally grown. Growth rates of those cultures exposed originally to the higher levels of radiation (20 and 40 mc/ml $H^3{}_2O$) were greatly different from that of control cultures throughout the 3-day period, but the growth rates of the other cultures paralleled that of the controls between the second and third day of subculture. Finally growth on subculture is nearly equal in per cent of mass of control cells, for those cells exposed to approximately equal doses of radiation from $P^{32}$ and $H^3{}_2O$ (3000 rep.; 116 μc $P^{32}$/ml, 10 mc $H^3{}_2O$/ml).

Growth rates of cells on subculture were a function of dose of radiation for cells grown in the presence of $Sr^{90}$-$Y^{90}$ and $S^{35}$. Mass of cells, in per cent of controls, was also nearly the same at 3 days of subculture for cells exposed to approximately 3000 rep. per day from $Sr^{90}$-$Y^{90}$ or $S^{35}$ as for cells exposed to the same level of radiation from $P^{32}$ or $H^3{}_2O$.

Studies were made of the average size, and numbers of cells per ml per log $I_o/I$ value of 0.60 at 660 mμ, of cells grown 3 days in the presence of tritium oxide. An increase in the average size of the cells following beta irradiation was characteristic. Typical results are given in table I.

TABLE I

THE EFFECT OF 40 MC/ML OF TRITIUM OXIDE ON THE DIAMETER, VOLUME AND NUMBER OF CHLORELLA CELLS

| Cells * | Average diameter μ | Average volume (calculated) $\mu^3$ | Number of cells per unit of volume † |
|---|---|---|---|
| Control ..... | 3.7 | 26 | $29 \times 10^6$ |
| 40 mc/ml .... | 5.7 | 97 | $8 \times 10^6$ |

* Grown for three days in the presence or absence of $H^3{}_2O$.

† Number of cells per ml per log $I_o/I$ (6600 A) of 0.60.

## DISCUSSION

Action of the ionizing radiations reduced the growth rate of the cells in proportion to the dose of radiation received. The reduction in growth rate was not evident, at the levels we used, until after the first day, but then the effect became progressively greater, and it persisted on subculture in non-radioactive media. Action of the ionizing radiations also resulted in an increase in cell size.

The results reported do not allow a deduction of the specific site or mode of action of the ionizing radiations on algae. A reduction in content of one or more enzymes below a growth-limiting level would, of course, reduce the growth rate of the cells, and the cells would not return to the normal rate until the enzyme content of the cell had returned to the growth-limiting level. This might take several generations. Likewise interference with cell division, or the gradual dropping out of lethal mutants over several generations would reduce the growth rate of the cells. Toxic products formed by living cells, or resulting from cell death, would also slow the growth rate of cells.

The increase in size of the algae during irradiation might result from an interference with cell division or from a change in the metabolism of the algae following changes in enzyme content. An increase in the content per cell of enzymes for starch synthesis and a decrease in content of other enzymes could result in an increased size of the cell if cell division is independent of the quantity of stored carbohydrate per cell. Likewise an enlarged size could result from damage to either the cell division mechanism or the enzymes synthesizing factors for cell division if the cell division process is relatively independent of the growth process.

It seems to us superfluous to speculate further on the sites of action of the beta-emitting isotopes in

Chlorella. Instead it is anticipated that further experimentation, now in progress, will yield data on biochemical and physiological sites in Chlorella which are most sensitive to damage by beta-emitting isotopes. We believe also that comparisons between the results we have obtained and those obtained by others with ultraviolet light (1, 2 and 5) would be meaningless. It is quite probable that the mechanisms of action, and the sites of damage, for these radiations are quite different. In general ultraviolet light brings about activation of those molecules which can absorb the light quanta, principally nucleic acids, whereas beta particles bring about ionization of molecules near the path of the particle. The latter action may be direct or mediated through products resulting from the ionization of water.

## Summary

Algae have been grown in the presence of several levels of $H^3_2O$, $P^{32}$, $Sr^{90}$-$Y^{90}$ and $S^{35}$ and then subcultured in non-radioactive inorganic nutrient solution. Growth rates of cells were decreased in proportion to the dose of radiation received. The reduced rate of growth continued in subculture as a function of the dose received. An increase in the average cell size also occurred during growth of the algae in the radioactive solutions.

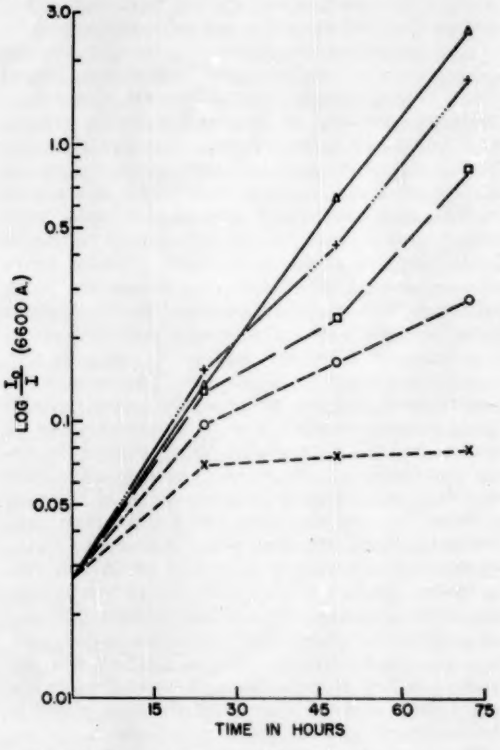


Fig. 3. The growth of Chlorella after exposure to tritium oxide. Inoculum grown in the presence of no $H^3_2O$—△; 5 mc/ml—+; 10 mc/ml—□; 20 mc/ml—○; and 40 mc/ml—×.

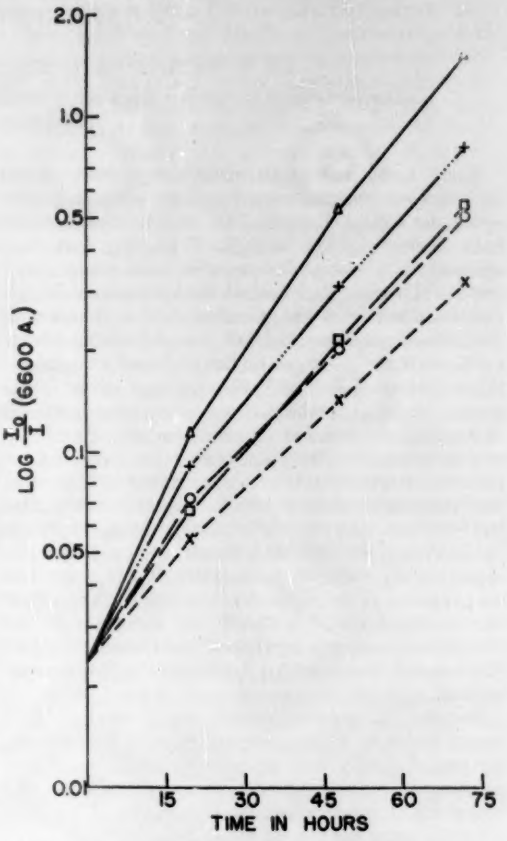


Fig. 4. The growth of Chlorella after exposure to phosphorus-32. Inoculum grown in the presence of no $P^{32}$—△; 39 μc/ml—+; 77 μc/ml—□; 116 μc/ml—○; 155 μc/ml—×.

The results reported do not allow a deduction of the specific site of action of the ionizing radiations on the algae.

Contributions of Dr. D. Weinberger and Mrs. M. S. Watson to this work are gratefully acknowledged.

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# EFFECT OF 2,4-DICHLOROPHENOXYACETIC ACID ON PHOTOSYNTHESIS AND RESPIRATION[1,2]

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Early work with plant growth regulators offered only indirect evidence regarding their effect on photosynthesis. Mitchell *et al.* (15) showed that starved bean leaves exposed to light after they had been sprayed with α-naphthaleneacetic acid accumulated less starch, sugar, and dextrin than unsprayed leaves, and thus indicated the possibility of a decrease in photosynthesis. Freeland (13, 14), measuring photosynthesis directly by gas exchange, found that 2,4-dichlorophenoxyacetic acid (2,4-D) and other plant growth regulators inhibited apparent photosynthesis in Anacharis and in bean leaves. In bean, 2,4-D in a concentration of 100 ppm caused an inhibition of photosynthesis amounting to approximately 20% over the 4-day experimental period; respiration was first inhibited and then stimulated during the same period. In Anacharis, 30 and 100 ppm of 2,4-D caused a decrease in the rate of photosynthesis, the reduction being greater at the higher concentration. Respiration was at first inhibited by 2,4-D, but showed a partial or complete recovery at the end of 48 hours. Rhodes (20) showed that 2-methyl-4-chlorophenoxyacetic acid reduced apparent photosynthesis of tomato plants.

Nickell (16) reported stimulation of respiration of tumor tissue of *Rumex acetosa* at very low concentrations of 2,4-D, with progressive inhibition of respiration at higher concentrations. Growth of the tumor tissue responded to the application of 2,4-D in much the same manner as respiration.

The dependence of the growth-promoting effect of naturally occurring auxin upon the concentration of the undissociated auxin molecule was established in 1934 (6), after Dolk and Thimann (12) had demonstrated that auxin activity was highest in an acid medium. This relationship has since been verified for synthetic plant growth regulators and other weak acids and bases by observing their effect in a variety of growth and respiration responses (2, 4, 5, 21, 22, 23, 25, 26). Albaum *et al.* (1) showed that the relation of the pH of the medium to the effect of externally applied indoleacetic acid on Nitella was due to the penetration of the indoleacetic acid, the coincidence of the curves for penetration of indoleacetic acid and its dissociation, plotted against pH, indicating that it entered as the undissociated acid.

Audus (3) found the inhibition of root growth to be proportional to the logarithm of the concentration of undissociated 2,4-D acid present, whether the range of concentrations of this molecule was achieved by varying the pH or by increasing the concentration at a constant pH.

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[2] Paper No. 775, University of California Citrus Experiment Station, Riverside, California.

The widespread use of 2,4-D in citrus culture for such purposes as increasing fruit size, delaying leaf and fruit abscission, and prolonging storage life of harvested fruit (24) has resulted in a situation in which a great deal of practical knowledge concerning the effects of the growth regulator on citrus is available, whereas little is known regarding the means by which 2,4-D alters the functioning of the plant to produce the observed responses. The present investigation is concerned with a quantitative study of the effect of 2,4-D on photosynthesis and respiration of citrus leaves and, for comparative purposes, its effect on the same processes in the unicellular green alga *Chlorella pyrenoidosa*.

## MATERIALS AND METHODS

EXPERIMENTS WITH CITRUS LEAVES: To reduce variation of the experimental material to a minimum, leaf samples were taken from a single Washington Navel orange tree growing in the field. The leaves selected were of a comparable physiological age, being those of the most recent growth flush, which had reached their full expansion and color development.

After harvesting, the leaves were brought into the laboratory in a closed container lined with moistened paper. For treatment and measurement of photosynthesis, 12 disks were cut from each leaf with a sharpened cork borer having an inside diameter of 8 mm. The 12 disks from each leaf were distributed among 12 beakers, 1 disk to each beaker. Six leaves were used for each run. The 6 disks in each beaker were then covered with solutions of buffer, or of buffer plus 2,4-D, prepared as described below. Circular pieces of stainless steel screen were placed over the leaves to ensure their remaining immersed in the solutions during the treatment. The beakers were then placed in a desiccator fitted with a stopcock and were subjected to a vacuum for 30 minutes. The vacuum was then released, and the infiltrated disks were removed from the solutions and placed on absorbent paper, to remain until the water-soaked appearance of the tissue disappeared, usually about 30 minutes. The disks were then transferred individually to 16-ml Warburg flasks having two side arms, the 6 disks from each treatment being arranged with their upper (non-stomate-bearing) surfaces downward around the center well of the flask so that there was no overlapping. One ml of a diethanolamine-carbonate buffer (18) of a composition that maintained a constant partial pressure of approximately 1% $CO_2$ in the flask was distributed, 0.3 ml to each side arm and 0.4 ml to the center well. A fluted filter paper was then placed in the center well, and the system was closed.

Measurements of photosynthesis, as evidenced by evolution of oxygen, were carried out in an illuminated

Warburg respirometer (27) at a temperature of 25°C, with a light intensity of approximately 1200 fc, for a period of 1 hour. A one-hour measurement of respiration in darkness completed the determination. The sum of the light and dark readings gave an approximation of true photosynthesis. The manometric assemblies were not shaken, thus avoiding the need for adding water or an adhesive to the flask to prevent the disks from overlapping during the determinations.

Buffer solutions of various $H^+$ concentrations were prepared by diluting a stock solution of phosphate buffer with water (for the controls) or with a solution of recrystallized 2,4-D acid brought to the desired pH by the addition of KOH, to give a final concentration of 0.04 M phosphate. The final concentration of 2,4-D in those buffers containing it was $2 \times 10^{-3}$ M. This procedure was used to minimize the change in pH of the solutions containing 2,4-D as a result of the increased $H^+$ concentration due to the acid.

For treatments with a range of 2,4-D concentrations at pH 4.5, solutions were prepared in the same manner with a concentrated phosphate buffer solution at pH 4.5 diluted with solutions of 2,4-D at the same pH to give a final phosphate concentration of 0.04 M and 2,4-D concentrations in the desired range.

The buffer concentration of 0.04 M was selected after preliminary experiments indicated that over the range of 0.01–0.05 M $PO_4$ there was very little effect of buffer concentration on the rate of photosynthesis of the leaves, whereas concentrations higher than 0.1 M inhibited the process appreciably, and those below 0.005 M did not have sufficient buffering capacity to resist changes in pH during the treatments.

To place the solutions in intimate contact with the cells and thus reduce to a minimum the time required for penetration into the cells, materials were applied to the leaves by infiltration. Day (10) has found that 2,4-D will penetrate through the cuticle and epidermis of bean leaves at approximately 30 $\mu$/hr. Under the conditions of the present work, this rate would more than ensure that 2,4-D would penetrate from the intercellular spaces into the cells before photosynthesis measurements were started. The fact that penetration had reached a maximum before measurements were begun was also indicated by the fact that there was no significant change in the rate of photosynthesis of any of the treatments during the period in which determinations were made. Leaf disks which were infiltrated with water alone or with 0.04 M phosphate buffer did not differ significantly in their rates of photosynthesis from noninfiltrated leaf disks.

The leaf-disk infiltration technique was used to eliminate as many as possible of the side effects of 2,4-D, such as an effect on translocation which would in turn change the rate of photosynthesis. The short period of exposure and the unnatural conditions prevailing during the treatment and during the measurement of photosynthesis do not allow direct comparison with field applications of the growth regulator, but do permit a more critical study of the effect on photosynthesis alone.

EXPERIMENTS WITH CHLORELLA: To eliminate inaccuracies resulting from failure to maintain a 2,4-D solution of known concentration in contact with the cells of the orange leaf, owing to change in concentration of the infiltrated solutions by water loss, and, particularly, to eliminate the possible effect of 2,4-D on stomate opening (7), a parallel series of experiments was run, using *Chlorella pyrenoidosa*.

The technique used for treatment and measurement of photosynthesis with Chlorella cells was of necessity somewhat different from that employed with the orange leaf disks. Chlorella was cultured aseptically in flasks containing 50 ml of nutrient solution. These flasks were shaken continuously in a greenhouse. After approximately 2 weeks, samples were removed into centrifuge tubes and centrifuged for 3 minutes. The cells were then resuspended in 3 ml of the buffer solution or of the buffer plus 2,4-D solution used for treatment and were transferred to Warburg flasks having two side arms. One ml of diethanolamine-carbonate buffer was then distributed in the side arms and center well, as indicated above. Photosynthesis and respiration were measured as in the preceding experiments except the flasks were shaken at 100 cycles per minute. Replications were obtained by successive runs using the same treatments.

DETERMINATION OF DISSOCIATION CONSTANT OF 2,4-D: The 2,4-D acid [3] used in these experiments was recrystallized twice from hot water, yielding odorless white crystals with a melting point of 138.6° to 139.6°C. The dissociation constant of this material was determined with the use of 0.003 and 0.004 M solutions. The hydrogen-ion concentrations of these solutions were measured with a Beckman model G pH meter after calibration with a solution of 0.05 M KH phthalate, the pH of which was taken as 4.005 (11). The $pK'_a$ value for 2,4-D acid, calculated from the equations

$$\frac{[H^+][A^-]}{[HA]} = K'_a;\ pK'_a = \log \frac{1}{K'_a},$$

was found to be 2.96. This compares with the values of 3.28 and 2.81–2.89 determined by Audus (3) and van Overbeek (26) respectively.

[3] Provided by The Dow Chemical Co.

## RESULTS

EFFECT OF HYDROGEN-ION CONCENTRATION ON INHIBITION OF PHOTOSYNTHESIS BY 2,4-D: The uppermost curve of figure 1 shows the effect of $H^+$ concentration, over the range from pH 3.10 to pH 8.05, on the photosynthesis of navel orange leaf samples infiltrated with 0.04 M phosphate buffer. There is a tendency for the highest rate to be achieved between pH 5 and 6, with the low point of the curve at the greatest $H^+$ concentration and no detectable difference between the rate at pH 7 and that at pH 8. The addition of $2 \times 10^{-3}$ M 2,4-D to the buffer solutions at the various pH values caused a change in the slope of the curve, as indicated in the second curve of figure 1. The continuing downward trend of this curve, from pH 8.05 to a low point at pH 3.10, indicates

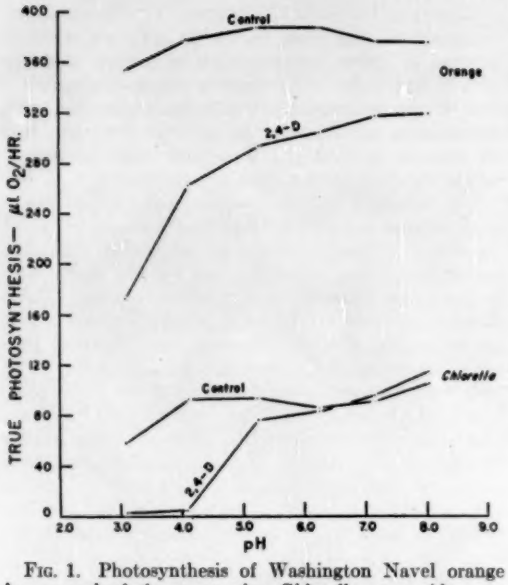


FIG. 1. Photosynthesis of Washington Navel orange leaves and of the green alga *Chlorella pyrenoidosa*, as affected by $H^+$ concentration, alone, and with the addition of $2 \times 10^{-3}$ M 2,4-D.

that with a decrease in pH the same concentration of 2,4-D caused an increasing inhibition of the process of photosynthesis.

The two lower curves of figure 1 show the effect of the same range of hydrogen-ion concentrations, with and without the addition of $2 \times 10^{-3}$ M 2,4-D, on the true photosynthesis of *Chlorella pyrenoidosa*. Photosynthesis was inhibited at pH 3.10 but showed very little effect of $H^+$ concentration over the rest of the pH range, with the possible exception of a tendency for a stimulation to occur above pH 7.

The 2,4-D curve shows a somewhat different effect than was found with the orange leaves. With Chlorella, inhibition was almost complete at pH 4.10 and 3.10, while at pH values above 7 the cells exposed to 2,4-D actually had a stimulated rate of photosynthesis, as compared with the controls.

Figure 2 represents the same data recalculated so that the percentage inhibition of photosynthesis by 2,4-D is plotted against the logarithm of the concentration of the undissociated acid present in each of the $H^+$ concentrations used. With both orange leaves and Chlorella, in the lower range of undissociated acid concentrations ($1.22 \times 10^{-7}$ to $9.55 \times 10^{-6}$ M, corresponding to pH 7.18 to 5.28), the data fit straight lines when plotted against the logarithm of the concentration of undissociated acid. The inhibition of photosynthesis in the orange leaves gives a line with the formula $Y = 17.80 + 3.17\,X$, with a correlation coefficient $r = 0.999$; in Chlorella, the line of best fit is $Y = -7.80 + 12.12\,X$, with a correlation coefficient $r = 0.906$. Attempts to fit the portions of the curves mentioned, and the entire range of concentrations, to an arithmetical relationship gave very low correlation coefficients. The higher range of undissociated acid concentrations ($9.55 \times 10^{-6}$ to $8.37 \times 10^{-4}$ M, corresponding to pH 5.28 to 3.10) failed to fit adequately either a logarithmic or an arithmetical regression.

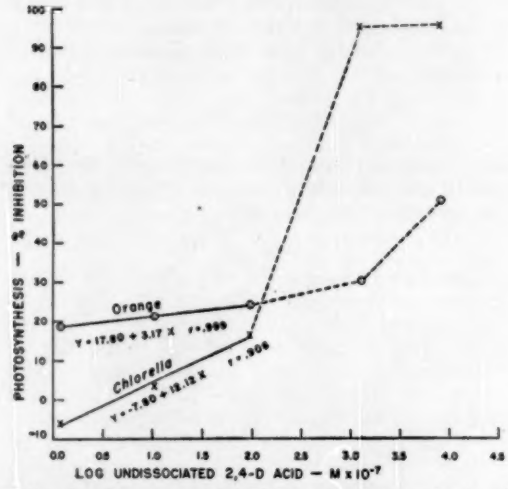


FIG. 2. Percentage inhibition of photosynthesis of Washington Navel orange leaves and of *Chlorella pyrenoidosa* by a range of undissociated 2,4-D acid concentrations obtained by varying the $H^+$ concentration of buffers containing a constant amount ($2 \times 10^{-3}$ M) of 2,4-D.

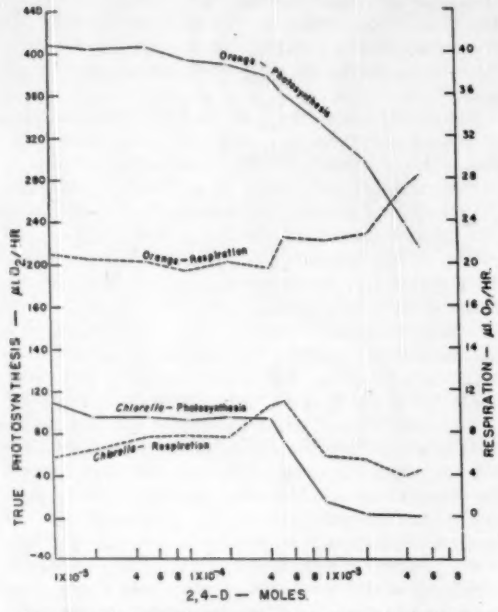


FIG. 3. Photosynthesis and respiration of Washington Navel orange leaves and of *Chlorella pyrenoidosa*, as affected by concentrations of 2,4-D from $1 \times 10^{-5}$ to $5 \times 10^{-3}$ M at pH 4.5.

These parts of the curves are indicated in the figure by means of dashed lines connecting the mean values.

EFFECT OF 2,4-D CONCENTRATION ON INHIBITION OF PHOTOSYNTHESIS: The effects of 2,4-D, in concentrations ranging from $1\times10^{-5}$ M to $5\times10^{-3}$ M at pH 4.5, on photosynthesis of navel orange leaves and of Chlorella cells are shown in figure 3. The values for the control treated with buffer at pH 4.5 (not shown on the chart) were 402 $\mu$l $O_2$/hr for the orange leaves and 108 $\mu$l $O_2$/hr for the Chlorella cells.

The fairly smooth curve for orange leaves when rate of photosynthesis is plotted against molar concentration of 2,4-D on a logarithmic basis, indicates a continuous decrease in rate of photosynthesis with increasing concentration.

In the case of the Chlorella cells there is very little effect of 2,4-D on photosynthesis over the range $1\times10^{-5}$ to $4\times10^{-4}$ M, with a rapid decrease brought about by an increase in concentration to $2\times10^{-3}$ M, at which point inhibition of photosynthesis appeared to be complete. Although photosynthesis was completely inhibited, the cells were apparently still alive, as evidenced by the fact that respiration continued, although oxygen uptake was at the same rate in both light and darkness. This oxygen uptake may, of course, have been due to some oxidative processes in nonliving cells.

Figure 4 shows the data of figure 3 plotted as percentage inhibition of photosynthesis against concentration of undissociated 2,4-D. In this case the inhibition of photosynthesis in both Chlorella and navel orange leaves by 2,4-D over the range of concentration of undissociated acid molecules from $5.4\times10^{-6}$ to $1.40\times10^{-4}$ M, fit straight lines calculated against concentration of undissociated 2,4-D. These curves—that of the orange leaves with the formula $Y = 4.38 + 3.01\,X$ and $r = 0.977$, and that of Chlorella with the formula $Y = 5.36 + 19.22\,X$ and $r = 0.924$—correspond

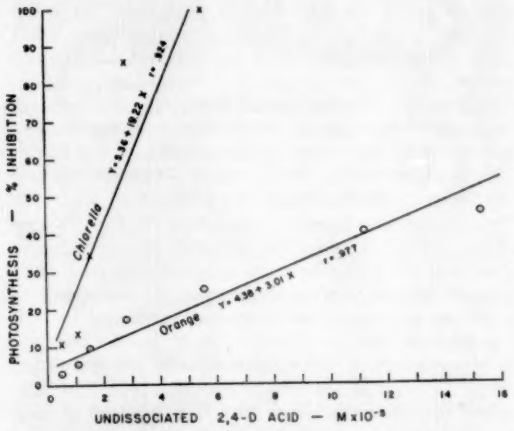


FIG. 4. Percentage inhibition of photosynthesis of Washington Navel orange leaves and of *Chlorella pyrenoidosa* by a range of undissociated 2,4-D acid concentrations obtained by varying the concentration of 2,4-D added to buffers at pH 4.5.

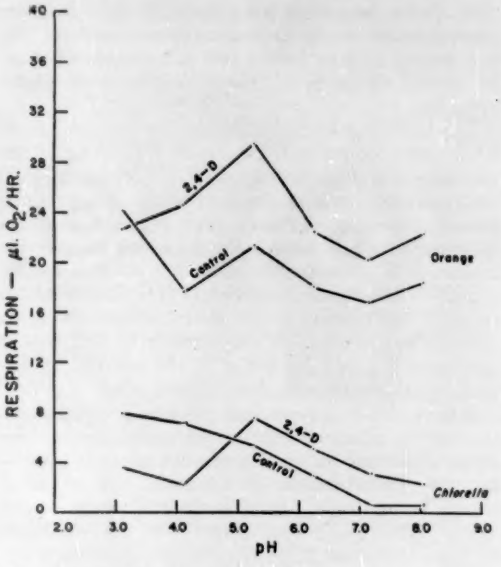


FIG. 5. Respiration of Washington Navel orange leaves and of *Chlorella pyrenoidosa*, as affected by $H^+$ concentration, alone, and with the addition of $2\times10^{-3}$ M 2,4-D.

to those portions of the curves in figure 2 which are drawn with dashes. They have good fit when calculated on an arithmetical basis and have low correlation coefficients when calculated on a logarithm of concentration basis.

EFFECT OF 2,4-D CONCENTRATION ON RESPIRATION: Respiration measurements made on the orange leaves and Chlorella cells at the time photosynthesis was determined are shown in figures 3 and 5. The curves in figure 3 show that with an increase in 2,4-D concentration at a constant $H^+$ concentration there is a tendency toward an increase in respiration. In the orange leaves this tendency continues to the highest concentration, but in Chlorella the stimulation is found only over a narrow concentration range and is followed by a progressive inhibition of respiration. Curves in figure 5 show that over the range from pH 3.10 to pH 8.05, there is inhibition of respiration in both orange and Chlorella only at the lower pH values, with a more or less constant stimulation of respiration in the higher pH values.

Since the mass of tissue used in these determinations was limited by the need for limiting the total oxygen evolution by photosynthesis to a value which could easily be determined in the Warburg apparatus, and since the respiration values are approximately one-tenth of those for photosynthesis, they are so low that it is difficult to draw valid conclusions from the small differences which occurred during these experiments.

## DISCUSSION

Although growth responses of plants to various growth substances, both naturally occurring and syn-

thetic, have frequently been shown to depend on the concentration of the undissociated molecule (2, 25), the present authors have found no previous reference to similar effects of a growth substance on photosynthesis.

The inhibition of photosynthesis by cyanide has recently been shown (17, 28) to be a function of the concentration of undissociated HCN. The concept of undissociated auxin as the controlling factor in pH-growth curves has recently been challenged on the basis that the pH of the bathing medium controls the ionization of plant proteins and thus controls the response to added growth regulators (8). Burström (9) suggests that calcium rather than undissociated auxin controls the response of root growth to pH, but he attributes this to a hardening of the cell wall by calcium, which counteracts the softening effect of auxin.

It nevertheless appears that the data presented here can best be interpreted by assuming that the undissociated 2,4-D molecule is the effective agent in inhibiting the photosynthesis of Chlorella and of navel orange leaves under the conditions of these experiments. This relationship seems to be best explained by the assumption that only the undissociated molecule penetrates (1) into the cell and thus is the effective form of the compound. These data indicate, however, that there may be at least two separate types of inhibition involved, and that, in addition, at least in orange leaves, part of the inhibition may be due to an indirect effect on photosynthesis through gas exchange, or to an effect of the ionized form of the acid.

The two sets of data presented here cover parts of the same range of undissociated acid concentration obtained in two different ways. The experiments summarized in figure 2 cover undissociated acid concentrations ranging from $9.05 \times 10^{-8}$ to $8.37 \times 10^{-4}$, while those in figure 4 range from $5.42 \times 10^{-6}$ to $1.40 \times 10^{-4}$, giving an expansion of a portion of the curves of figure 2. As shown in figure 2, only the lower concentration range can be fitted significantly to the logarithm of undissociated 2,4-D concentration, with a sharp break in the curve for Chlorella and a less obvious change for navel orange leaves at a value corresponding to $9.50 \times 10^{-5}$ M of undissociated acid.

Considering only the portion of the curve (fig. 2) which is presented as a solid line, the relationship indicates the possibility of an effect of 2,4-D on some rate-limiting enzyme reaction concerned with photosynthesis, making the rate of photosynthesis inversely proportional to the logarithm of undissociated 2,4-D acid. Since the undissociated acid is assumed to be the form which penetrates, the concentration within the cells depends on the concentration of undissociated acid in the medium, and the slope of the curve thus represents the proportionality of reacting molecules or the affinity of 2,4-D for the substance with which it is reacting. The difference in slope of the orange leaf curve might be due either to a lower effective concentration within the cells as a result of the different method used for treatment, or to a greater adsorption of 2,4-D by some nonactive material within the cells (8), or to a combination of these two with other factors.

The intercept of the Chlorella curve indicates a stimulation of photosynthesis by the lower range of concentrations of 2,4-D, but the same proportionality would not necessarily be carried through the concentration range lower than that used here. The intercept of the orange leaf curve indicates an appreciable inhibition at extremely low concentrations of undissociated 2,4-D. This might be due to one or more of a number of factors, such as closure of the stomates, which has been shown to be one of the effects of 2,4-D application (7, 19), or to an effect of the dissociated form of the growth regulator such as has been postulated (8, 23), or, more likely, to a different level of undissociated acid within the cell, governed by the pH of the cell itself. The continuous bathing of the Chlorella cells in the solution would render them likely to undergo a greater change in their internal pH than the orange leaves. Due to lack of any means for accurate determination of the internal pH of the cells, the question of whether or not the undissociated acid is the only form which reacts within the cells, as well as the only form which penetrates into the cell, cannot be settled, and effects which might arise out of this relationship must remain speculative.

The portions of the two curves which do not fit the same relationship as those discussed above, and which are indicated in figure 2 by dashed lines, are thought to represent a different type of inhibition. A part of the same concentration range used in figure 2 is covered by the curves in figure 4, where the different concentrations of undissociated acid were obtained at a constant pH by a change in total concentration of 2,4-D. This range, from $5.42 \times 10^{-6}$ to $1.40 \times 10^{-4}$ M of undissociated acid, corresponds approximately to the range of log concentration of undissociated acid in figure 2 from 2.0 to 3.0. In this case, the data can be fitted most accurately to an arithmetical relationship in which the inhibition of photosynthesis is dependent on the concentration of undissociated 2,4-D acid. Here again the curve for Chlorella has a much steeper slope than the one for orange leaves, but in this case the intercepts are rather close, and both fall near zero. The area in which the curves for the two types of inhibition cross is rather indefinite, the inhibition being somewhat less in figure 4 than in figure 2 for the same concentration of undissociated acid. Investigations are presently underway to give further experimental points for this region of the curve and to elucidate the role which the hydrogen ion itself may play in determining the response of photosynthesis to 2,4-D through an effect on the dissociation of the cell constituents (8).

Observations of Chlorella cells after the conclusion of photosynthesis and respiration determinations indicated that the cells which had been exposed to concentrations of undissociated 2,4-D higher than $9.35 \times 10^{-5}$ M were progressively decolorized by higher concentrations, the difference being obvious to the unaided eye, with a complete lack of green color in those cells in which photosynthesis had been completely inhibited.

This suggests that the second type of inhibition being dealt with here, over the range from $4.52 \times 10^{-6}$ to $1.36 \times 10^{-4}$ M of undissociated acid, may be due to a progressive destruction of chlorophyll, or to inhibition of chlorophyll synthesis by the 2,4-D, resulting in a decreased photosynthesis.

## Summary

The effect of 2,4-dichlorophenoxyacetic acid on photosynthesis and respiration of leaves of Washington Navel orange and of the unicellular green alga *Chlorella pyrenoidosa* has been studied.

The inhibition of photosynthesis by 2,4-D is shown to be related to the concentration of undissociated 2,4-D acid molecules in the solution used for treatment, regardless of whether the difference in undissociated acid concentration was obtained by varying the pH of buffer solutions containing a constant amount of 2,4-D, or by varying the concentration of 2,4-D added to buffers of the same pH.

Two phases of inhibition are shown: one, in a low range of undissociated acid concentration, is proportional to the logarithm of this concentration; the other, covering a higher concentration range, is proportional to the concentration of undissociated 2,4-D and is thought to represent a direct or indirect destruction of chlorophyll by the 2,4-D.

The effect of the same concentrations of 2,4-D on respiration of the plant tissues was slight, but tended to show stimulation in intermediate ranges of undissociated acid concentration and inhibition in the higher concentration ranges.

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# ORGANIC ACID METABOLISM AND ION ABSORPTION IN ROOTS[1, 2]

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It is axiomatic that the total charge of ions inside plant cells must be electrically equal to zero. Nevertheless, preferential absorption or uptake of ions by plants from external environments can and does occur. Since roots may absorb cations and anions at unequal rates from single salt solutions, they must therefore have at their disposal mechanisms for maintaining cation-anion equivalency. In recent years, the role of organic acid metabolism in relation to the maintenance of cation-anion balance have been investigated by Pucher, Vickery and Wakeman (15), Ulrich (17), Overstreet, Broyer, Isaacs and Delwiche (11), Pierce and Appleman (13), Burström (2), Cooil (5), and others.

Ulrich (17), working with excised barley roots, concluded that when excess cations were absorbed, "organic acids were formed as a response to the tendency toward an increase in pH of the root sap. Conversely, when excess anions were absorbed, organic acids tended to disappear leaving the base to balance the increase of inorganic anions." He further concluded that the organic acids were associated with respiration directly or indirectly.

On the basis of buffer curves of plant saps and fractional precipitation with barium salts in alcoholic solution, Burström (2) tentatively concluded that the organic acid fraction from wheat sap consisted largely of malic acid with small amounts of other acids, mostly citric. The citric acid in no case constituted more than 10% of the total acid. Burström states that the buffer system of the wheat plant consists of the formation or destruction of malic acid so its content approximately equals the existing cation excess, and the regulation of the pH of the cell sap by the buffer action of $CO_2$. In a later paper, Burström (3) relates the role of organic acid in ion balance to the Lundegärdh scheme of anion respiration.

A valid theory of ion absorption should be consistent with all observed changes occurring in the ionic composition of the absorbing tissues. Thus, to study the mechanisms of ion absorption, it becomes highly desirable to examine the nature of the changes in ionic balance during absorption.

Since organic acids play an important role in the cation-anion balance, it likewise becomes imperative to conduct a thorough survey of the organic acids which occur in the absorbing tissue and to follow their changes during the course of absorption. This has now become readily feasible with the recent application of quantitative chromatographic techniques to organic acid analysis.

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[2] This paper is based on work performed under contract No. AT-(11-1)-34, project 5, with the Atomic Energy Commission.

## MATERIALS AND METHODS

All experiments were carried out with excised roots, usually from 6-day-old barley seedlings (Sacramento variety). Seedlings were grown in a very dilute nutrient solution in the dark as earlier described (7). Also, low-salt roots were obtained from plants grown in the greenhouse in ⅛ strength Hoagland nutrient solution.

The excised roots were rinsed thoroughly in distilled water and centrifuged for five minutes at approximately $65 \times g$. Weighed portions of roots were then placed into the various solutions. All absorption experiments were carried out at 26°C. In most experiments, 15 gm of excised root material were allowed to absorb from 15 liters of solution. In no case were more than 26 gm of roots used in 15 liters of solution. A large ratio of solution to root weight was used to minimize concentration changes in the culture solution. All solutions were aerated during the experiment. After the absorption period, the treated roots were carefully washed and stored at −18°C until lyophyllized. Roots to be used for organic acid fractionation were lyophyllized immediately after treatment.

Potassium was determined in ashed aliquots of the dried and ground root material by precipitation as the cobaltinitrite, and subsequently titrated with ceric sulfate. Calcium was precipitated as the oxalate and titrated with ceric sulfate. Bromide was determined by the iodometric procedure of White and Kilpatrick (18) after fusing a suitable aliquot of the dried sample with NaOH in a nickel crucible. Calcium plus magnesium were estimated together by a method essentially similar to the ethylenediamine tetraacetate titration procedure of Cheng and Bray (4); nitrate as described by Johnson and Ulrich (9); inorganic sulfate according to Johnson and Nishita (8); ammonia by the method of Pucher, Vickery and Leavenworth (14); and total organic acid, malic, citric and oxalic acids by the methods of Pucher, Wakeman, and Vickery (16). Fractionation of the organic acids was accomplished through the use of silica gel chromatography as described by Bulen, Varner, and Burrell (1). Inorganic phosphate in the lyophyllized root material was determined colorimetrically by the molybdenum blue reaction.

## RESULTS

BARLEY SEEDLINGS ROOTS: When ions are absorbed by plant roots, a) equivalent amounts of cations and anions are absorbed, or b) excess cation, or c) excess anion absorption occurs from the external solution. The first condition was examined by allowing excised roots from 6 day old barley seedlings to absorb from 0.005 *N* KBr solutions for periods up to 3 hours. The

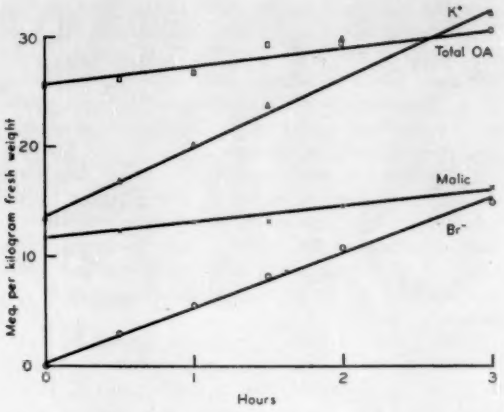


FIG. 1. Potassium, bromide, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* KBr, as a function of time.

absorption rates of potassium and bromide from KBr are not very different for this root material. The treated roots were analyzed for K, Br, total organic acid and malic acid, as shown graphically in figure 1. In 3 hours, the roots had absorbed 18.9 meq potassium and 15.4 meq Br per kg fresh weight indicating 3.5 meq excess cation absorption. In the same period the total organic acids increased by 5.2 meq per kg fresh weight. The malic acid increased by 4.6 meq. In this case, the increase in organic acid anion more than compensated the relatively small cation excess and therefore other changes in the root ions were involved to a small extent. The pH of the expressed sap of the barley seedling roots (i.e., 5.7 to 6.0) in this, as well as in the following experiments, is such that the organic acids are almost completely dissociated.

As an example of the second case, the experiments of Overstreet, Ruben and Broyer (12) with radioactive carbon suggested that the absorption of potassium from $KHCO_3$ was largely excess cation absorption and this view is supported in the following experiment. An experiment carried out as described above, but in 0.005 *N* $KHCO_3$, gave the K, organic acid and malic acid results plotted in figure 2. The excess cation absorption (K) was 20.6 meq per kg fresh weight. The increase in total organic acids and malic acid were 18.9 and 19.8 meq respectively. In this instance, the increase in total organic acid anion very nearly approached the excess cation absorbed. The apparently greater production of malic acid than of total organic acid may or may not be significant. Some data reflecting on this point will be described later.

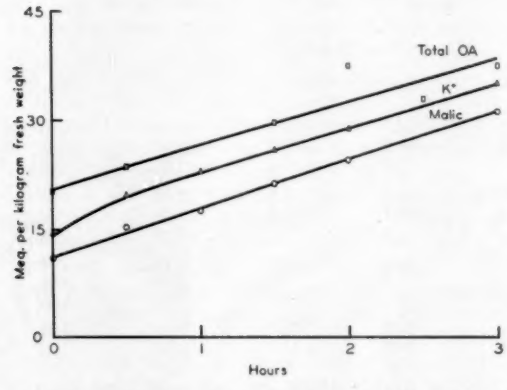


FIG. 2. Potassium, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* $KHCO_3$, as a function of time.

The third case of differential absorption of ions is shown with $CaBr_2$. Roots were placed in 0.005 *N* $CaBr_2$ for various periods up to 3 hours. The Ca, Br, total organic acid and malic acid data are plotted in figure 3; 3.5 meq calcium per kilogram fresh weight and 8.6 meq bromide were absorbed in 3 hours, producing an excess anion absorption of 5.1 meq. During this period, the roots lost 3.7 meq total organic acid and 2.8 meq malic acid. The decrease in total organic acid anion was not sufficient to compensate entirely for the excess absorption of bromide over calcium, the discrepancy amounting to 1.4 meq. However, similar discrepancies amounting to as much as 7.0 meq have been obtained in other experiments.

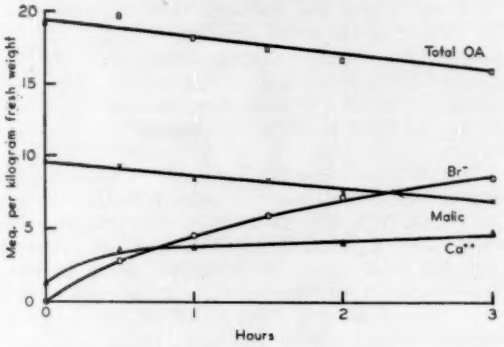


FIG. 3. Calcium, bromide, total organic acid and malic acid contents of excised barley seedling roots placed in 0.005 *N* $CaBr_2$, as a function of time.

In a modified experiment, excised barley roots were allowed to absorb from 0.005 *N* $CaBr_2$ for 3 hours and the treated roots then transferred to distilled water for 3 hours. This was done in order to observe any changes in the organic acid content following the initial treatment. The Ca, Br, total organic acid and malic acid levels were followed over the entire period of the experiment. The values are presented in figure 4. At the end of the $CaBr_2$ treatment, the roots had absorbed a bromide excess of 6.2 meq per kg fresh weight over the Ca absorbed. The total organic acid anion at this point had decreased by 3.7 meq, leaving an unaccounted excess of 2.5 meq of Br. Upon placing the treated roots into distilled water, some Ca and Br were lost from the roots and at the same time, the

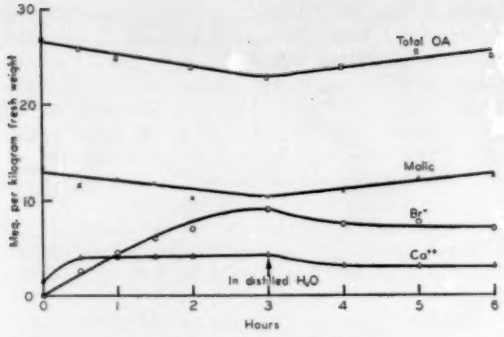


FIG. 4. Calcium, bromide, total organic acid and malic acid contents of excised barley seedling roots as a function of time. Roots placed in 0.005 *N* $CaBr_2$ for periods up to 3 hours. 3 hour treated roots then transferred to distilled water for periods up to an additional 3 hours.

total organic acid and malic acid increased somewhat. By comparing the initial and final values, it will be observed that for the entire experiment, the roots had gained 5.5 meq more bromide than calcium. The total change in organic acid anion was a decrease of 1.2 meq leaving 4.3 meq of Br to be compensated by other means.

MATURE PLANT ROOTS: The plants listed in table I were grown in dilute nutrient media in the greenhouse. All plants, with the exception of Atlas barley, were cultured for a period of 4 or 5 weeks before harvesting. For Atlas barley, the period was about 2 weeks. Excised roots from these plants were allowed to absorb from solutions of 0.005 *N* $KHCO_3$ for 3 hours. Analyses were made on the roots, before and after treatment, for K, Ca plus Mg, total organic, malic, citric, and oxalic acids. These data are given in table I. For every root sample reported in the table, Ca plus Mg was lost from the roots at the same time as K was being absorbed. Roots from Romaine lettuce lost nearly as much Ca plus Mg as K absorbed. In this case, the total organic acid increased but little. With the exception of the above material, poor agreement was obtained between the change in total organic acid content and the changes in the K and Ca plus Mg contents. It would appear that other ions were involved to a marked extent or that changes within the so-called total organic acid fraction were occurring. Some evidence of the latter is seen in the peculiar situation of having malic acid increasing in all the root material by an amount larger than that of the total organic acid.

ACETIC ACID IN BARLEY SEEDLING ROOTS: Initially, in order to fractionate the organic acids, they were extracted by a modification of the method of Isherwood (6). The ground material was acidified with $H_2SO_4$, mixed with silica gel and eluted with 50% (v./v.) butyl alcohol-chloroform. The eluted acids were re-extracted from silica gel with 50% (v./v.) tertiary amyl alcohol-chloroform. However, the total organic acids extracted by this procedure were invariably lower than when extracted by the method of Pucher, Wakeman and Vickery (16). For untreated barley seedling roots, the Isherwood procedure resulted in values which were only about 60% of those obtained by the Pucher *et al.* method. For roots, treated with 0.005 *N* $KHCO_3$ for 3 hours, the comparison was better. About 80% was recovered by the Isherwood procedure. Since the total organic acid (Pucher *et al.* method) had doubled as a result of the $KHCO_3$ treatment, it appeared that there might be an approximately constant amount of some acid present which was extracted by the Pucher *et al.* method, but not by the Isherwood procedure. Such was indeed found to be the case and the acid in question was identified as acetic acid. Initial identification was established through fractionation on silica gel columns and by

TABLE I

THE EFFECT OF 0.005 *N* $KHCO_3$ FOR 3 HOURS ON THE CONTENT OF POTASSIUM, CALCIUM PLUS MAGNESIUM, TOTAL ORGANIC, MALIC, CITRIC AND OXALIC ACIDS OF THE EXCISED ROOTS FROM MATURE PLANTS

| | | MEQ PER KILOGRAM FRESH WEIGHT | | | | | |
|---|---|---|---|---|---|---|---|
| | | K | Ca + Mg | TOTAL OA | MALIC | CITRIC | OXALIC |
| *Hordeum vulgare* (Atlas 46 barley) | Initial | 34.1 | 21.2 | 53.7 | 33.4 | 0.77 | 0.98 |
| | Final | 62.3 | 16.5 | 64.4 | 50.5 | 2.44 | 0.98 |
| | Change | +28.2 | −4.7 | +10.7 | +17.1 | +1.67 | 0.00 |
| *Soja max* (Hawkeye soybean) | Initial | 38.8 | 28.5 | 50.3 | 6.7 | 1.21 | 0.68 |
| | Final | 56.8 | 23.4 | 60.7 | 17.8 | 1.41 | 0.56 |
| | Change | +18.0 | −5.1 | +10.4 | +11.1 | +0.20 | −0.12 |
| *Cucurbita maxima* (Early white squash) | Initial | 16.7 | 34.7 | 28.0 | 4.3 | 2.17 | 2.47 |
| | Final | 30.7 | 29.2 | 30.1 | 8.9 | 2.38 | 0.81 |
| | Change | +14.0 | −5.5 | + 2.1 | + 4.6 | +0.21 | −1.66 |
| *Medicago sativa* (Chilean alfalfa) | Initial | 17.6 | 61.1 | 61.2 | 22.8 | 13.6 | 0.51 |
| | Final | 29.2 | 52.2 | 58.8 | 22.8 | 12.6 | 0.00 |
| | Change | +11.6 | −8.9 | − 2.4 | 0.0 | −1.0 | −0.51 |
| *Lactuca sativa* (Romaine lettuce) | Initial | 15.3 | 76.2 | 85.7 | 19.8 | 7.85 | 48.2 |
| | Final | 26.1 | 67.1 | 86.8 | 21.4 | 8.34 | 49.0 |
| | Change | +10.8 | −9.1 | + 1.1 | + 1.6 | +0.49 | +0.8 |

paper chromatography. Final identification was accomplished by a Du Claux distillation. In all cases, the correspondence between the unknown acid and acetic acid was exact.

Some preliminary studies of the acetic acid fraction indicate that this acid is not present in the free form in the barley seedling roots. The free acetate is present only in small amounts. The "bound" acetic acid is insoluble since it remains in the residue after extraction of ground root material with water. It is, however, readily hydrolyzed by treatment with 0.1 *N* $H_2SO_4$ on the steam bath for several hours. After such treatment, all of the acetic acid can be recovered in the free form. The expressed sap from frozen roots also contain but little acetate, free or "bound," the bulk of the acetate remaining in the press cake.

Organic Acid Fractionation: Samples of excised roots from barley seedlings were permitted to absorb for 3 hours from 0.005 *N* solutions of KBr, $CaBr_2$, and $KHCO_3$, and a control sample was placed in distilled water. The pH of the KBr solution was adjusted to 4.95, since at this pH and with this material, the rates of uptake of K and Br from KBr are almost exactly equal. After the absorption period, the samples were washed, lyophyllized and ground. The percentage dry weight of the samples varied from 5.85 to 6.1%. The total organic acids were extracted from suitable aliquots of the ground material by the ether extraction procedure as described by Pucher, Wakeman and Vickery. The individual organic acids were isolated from this fraction by the silica gel column fractionation procedure of Bulen, Varner and Burrell (1). The distribution of the acids in the effluent fractions were mapped out by running various combinations of known acids. When sufficient material was available, the identity of the isolated root acids was further confirmed through the use of paper chromatography.

The analyses of the initial and treated root samples are given in table II. In addition to the organic acid analyses, data are presented for the major inorganic ions. Acetic acid appears in the organic acid fraction since it is liberated by the extraction procedure. However, it is probably not available for cation compensation and so is not included in the cation-anion balance calculations. Other organic acids than those listed were either absent or present in too small a quantity to detect. The list of acids appears to be fairly complete since their sums check quite well with the corresponding total organic acid values as obtained by the titration procedure for total acids as described by Pucher *et al.* From 93.2 to 94.8% of the total acid values were recovered as isolated acids. This is good agreement with the average recovery figure for the pure acids adsorbed on the silica gel column. Two unidentified acids were present, acids A and B in table II. Both were present in small quantity, the former in all samples and the latter primarily in the $KHCO_3$ treated roots. Only the trans form of aconitic acid was detected. However, the treatment used to isolate the acids would have been sufficiently strong to have converted any cis-aconitic acid to the trans form.

Although most of the acids increased during excess cation absorption, malic acid and to a considerably lesser extent succinic acid accounted for most of the increase. The major decrease in organic acid content during excess anion absorption was accounted for by a decline in the malic acid content. Changes in the organic acids of the roots during the distilled water treatment were relatively small. This was also true when conditions of equivalent cation and anion uptake prevailed.

## Table II

The Effect of Water and 0.005 *N* Solutions of KBr, $CaBr_2$ and $KHCO_3$ for 3 Hours on the Organic Acid Composition and Ion-balance in Excised Barley Seedling Roots

| | Treatment | | | | |
|---|---|---|---|---|---|
| | Values in meq per kilogram freshweight | | | | |
| | Initial | $H_2O$ | KBr | $CaBr_2$ | $KHCO_3$ |
| *Organic acids* | | | | | |
| Acetic | 8.13 | 8.00 | 9.32 | 8.83 | 9.40 |
| Pyruvic * | 0.079 | 0.280 | 0.053 | 0.265 | 0.441 |
| Fumaric | 0.588 | 0.511 | 0.518 | 0.485 | 0.626 |
| Succinic | 0.423 | 0.407 | 0.474 | 0.428 | 2.42 |
| Trans-aconitic * | 0.391 | 0.396 | 0.301 | 0.458 | 0.653 |
| Oxalic | 0.352 | 0.228 | 0.260 | 0.327 | 0.322 |
| Malic | 11.80 | 13.80 | 13.20 | 8.31 | 29.31 |
| A † | 0.276 | 0.315 | 0.243 | 0.209 | 0.181 |
| B † | 0.000 | 0.030 | 0.039 | 0.000 | 0.179 |
| Citric | 0.216 | 0.359 | 0.368 | 0.258 | 0.632 |
| Total | 22.25 | 24.33 | 24.78 | 19.57 | 44.16 |
| Total OA (Pucher *et al.* method) | 23.8 | 25.7 | 26.5 | 20.7 | 47.3 |
| *Anions* | | | | | |
| Total OA-acetic | 15.7 | 17.7 | 17.2 | 11.9 | 37.9 |
| $Br^-$ | 0.0 | 0.0 | 17.5 | 7.8 | 0.0 |
| $NO_3^-$ | 1.0 | 1.4 | 0.2 | 0.1 | 1.1 |
| $SO_4^=$ | 3.6 | 3.4 | 3.1 | 3.0 | 3.6 |
| $H_2PO_4^-$ | 4.8 | 4.2 | 4.1 | 4.8 | 4.4 |
| Total anion | 25.1 | 26.7 | 42.1 | 27.6 | 47.0 |
| *Cations* | | | | | |
| $K^+$ | 14.6 | 14.9 | 32.0 | 14.7 | 38.5 |
| $Ca^{++} + Mg^{++}$ | 9.8 | 8.6 | 7.7 | 11.8 | 8.2 |
| $NH_4^+$ | 1.5 | 2.4 | 2.2 | 2.9 | 1.2 |
| Total cation | 25.9 | 25.9 | 41.9 | 29.4 | 47.9 |

* Identification tentative.
† Unidentified.

Measurements of pH made on the expressed sap of frozen roots, showed the changes for the various treatments to be small, varying from a low of 5.73 for the water treated sample to a high of 6.02 for the $KHCO_3$ treated sample.

## Discussion

Cation and anion equivalency is accomplished by absorption, and exchange of mineral ions, and by metabolic gains or losses of ions within the root. When absorption takes place from salts in which

cation and anion absorption rates are equal, no problem exists with respect to cation-anion balance.

If the rate of cation absorption exceeds that of anion absorption, the roots either lose cations of different species to the external solution or through synthesis make available anions within the roots. In roots of older plants, the exchange of cations may be of considerable magnitude as illustrated by Romaine lettuce, where as much as 84% of the K taken up from $KHCO_3$ exchanged for Ca plus Mg (table I). On the other hand, only a small amount of exchange occurred in barley seedling roots (table II). This difference in behavior may be related to the age of the plant or to the much greater initial calcium plus magnesium content of the more mature plant roots (21.2 to 76.2 meq per kg fresh weight) as compared to the barley seedling roots (9.8 meq). It appears that in the older plant material, the amounts of Ca plus Mg exchanging out are proportional to their initial concentrations. Presumably other cations could participate in this exchange reaction in a similar manner.

It has been pointed out by Jacobson, Overstreet, King and Handley (7) that the overall result of the postulated absorption reaction, $HR + M^+ = MR + H^+$, is the apparent exchange of a hydrogen ion from the root for a cation present in the external solution. However, this is not an exchange reaction in the same sense as that described above. If there were a simple exchange of pre-existing $H^+$ within the roots for external cations, the pH of the root sap should rise markedly during excess cation absorption in view of the weak buffering capacity of roots at the higher pH's (2). This does not occur. Evidently the hydrogen becomes available in ionic form only during the actual absorption reaction.

The other important mechanism for compensating excess cation absorption is the formation of organic acid anions within the cell. This is particularly marked in the case of the barley seedling roots. In this material, and probably in the roots of the more mature plants, malate is the most important acid radical participating in cation balancing. It is likely that the increases in the other organic acid radicals are caused by the accumulation of malate. An anomalous situation appears to exist for the samples reported in table I, since the increase in malate is greater than that of the total organic acid (particularly so in the case of Atlas barley) and for most samples, the increase in total organic acid is insufficient to compensate the excess of cations after deducting the calcium plus magnesium exchanged. It is possible that some anion other than an organic acid radical is involved. For example, Cooil (5) has shown that bicarbonate in guayule leaves can function in cation balancing. In any event, this would not explain the disproportionate increase of malate over total organic acid. If these root samples contain "bound" acetic acid, either a disappearance or conversion to a free acid, during the course of K absorption from $KHCO_3$ solutions could account for both the apparent anion deficiency and the apparent excess malate increase over total organic acid increase. However, it must be pointed out that such is not the case in barley seedling roots where the "bound" acetic acid increases somewhat as a result of the $KHCO_3$ treatment.

For excess anion absorption to occur, excised roots must either produce cations within the root or lose pre-existing anions. The data of table II indicate that $NH^+_4$ increases during the $CaBr_2$ treatment. However, major compensation of the excess anion absorbed is afforded by the loss of anions in the roots. A small amount of inorganic anion is lost, most probably through direct exchange, although possibly by metabolic conversion to a non-ionic form. Much of the decline in initial root anions occurs in the malate content. It is conceivable that malate could exchange for the external anion, but the increase in R.Q. values obtained by Ulrich (17) during excess anion absorption under similar conditions suggest that malate is more likely being lost via metabolism.

The close parallelism between malate synthesis and excess cation absorption as well as the absence of any significant delay in its production strongly suggest that in barley seedling roots, malate is the first organic anion of appreciable stability to be formed during the absorption of excess cations. Burström (3) has suggested that the malate is accumulated as an intermediate of the acid cycle of respiration. He further suggests that an equilibrium exists between malic acid and malate whose value is dependent upon the prevailing pH. When excess cations are present, the dissociation shifts in favor of the malate and "an amount of malate equivalent to the cation excess is withdrawn from the respiration cycle, and is apparently excreted together with the cations into the vacuole."

In connection with this suggestion, it should be noted that malate could not accumulate appreciably as a result of the normal functioning of the tricarboxylic acid cycle since it is a member of the cycle. However, if any member of the tricarboxylic acid cycle (other than acetic acid) were produced by some reaction external to the cycle, it could then use a portion of the cycle as a pathway for the synthesis of the malic acid and thus lead to the accumulation of malate. Among other possibilities are the synthesis of malic acid by reductive carboxylation of pyruvate via malic enzyme and the deamination of aspartic acid.

As yet, there is no evidence indicating the significance of malic acid in the actual absorption mechanism. The synthesis of malic acid may be an additional mechanism which assumes an active role only when excess cation absorption occurs or it may be produced even when equivalent absorption of cation and anion occurs. In the latter case, it would necessarily be metabolized as rapidly as formed. That is, the formation of the malic acid could be a normal consequence of cation absorption and the disappearance a normal consequence of anion absorption. According to the views of Lundegärdh (10) and Burström (3), the accumulation of organic acid radicals is caused by the deficiency of anion absorption rather than by the absorption of excess cations.

By suitable treatment with dilute acid, the rather impressive amount of 0.8 to 0.95% acetic acid, on a dry weight basis, can be obtained from barley seedling roots. Acid treatment such as used in the Isherwood procedure for total organic acid extraction (i.e., pH 2 for 2 to 3 hours at room temperature) is not sufficient to liberate the acetic acid. On the other hand, treatment at pH 0.5 for 16 to 18 hours at temperatures somewhat above room temperature (Pucher, Wakeman and Vickery method) appears to completely liberate the "bound" acetic acid from its insoluble form.

At present, the role of the "bound" acetic acid in the ion absorption process or in metabolism in general is obscure. As yet, little can be said concerning the form in which the acetic acid is bound or even if it exists as acetic acid. The acid could arise as a decomposition product although the relatively mild treatment required to liberate it makes this unlikely. Preliminary analyses for organic phosphorus and organic sulfur indicate that these elements are not involved in the acetic acid binding since they are present in smaller molar quantities than the acetic acid.

## Conclusions

During the absorption process, ionic balance is maintained in excised roots by absorption, by exchange for previously absorbed ions or by metabolic changes within the root, the latter being confined largely to the malic acid fraction. When excess cation absorption occurs, exchange for previously absorbed cations and the production of malate are the most important means of ion compensation. The exchange reaction is particularly significant in older roots, whereas the synthesis of malate predominates in barley seedling roots. When excess anion absorption prevails, much but not necessarily all of the compensation is due to a decrease in malate content.

The accumulation, and presumably the loss, of malate which may occur during the ion balancing process cannot arise via the normal functioning of the tricarboxylic acid cycle since malate is a member of the cycle. To account for the accumulation of malate, it is necessary to assume that either larger amounts of malate are synthesized only when excess cations are absorbed or that malate is utilized at a slower rate under conditions of decreased anion absorption.

Acid treatment of barley seedling roots results in the liberation of nearly 1% acetic acid on a dry weight basis. In untreated barley seedling roots, this acid constitutes about 35% of the total organic acid fraction as determined by the ether extraction procedure.

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# ZINC CONTENT OF BEAN PLANTS IN RELATION TO DEFICIENCY SYMPTOMS AND YIELD [1, 2]

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Zinc deficiency has been reported in beans (*Phaseolus vulgaris*) grown on peat soils in Florida (5), old corral areas in the San Joaquin Valley, California (3), and more recently on newly irrigated soils in Washington (6, 7). Recommendations for control by foliage applications of $ZnSO_4$ were made in each case.

Sommer (4) found no distinctive symptoms of zinc deficiency in the Red Kidney variety grown in low-zinc solution cultures until flowering, at which time there was rapid abscission of leaves and flower buds. The reports cited above on field-grown plants show that distinctive symptoms of deficiency are apparent even in early growth stages.

In Washington the first symptom on the Red Mexican variety is a mild general chlorosis of the leaves which may be seen even on the expanding cordate leaves if the deficiency is severe. Subsequent stages are interveinal chlorosis, followed by necrosis and browning of spots in the leaf mesophyll. The lower, older leaves are more severely affected than the young terminal growth. Abscission of flowers and older leaves may occur. Few flowers set seed, and the beans which develop are small and mature slowly. Typical Zn deficient plants are shown in figure 1.

No data on zinc contents of bean plants in relation to deficiency symptoms and response to zinc treatments are available. The purpose of this paper is to supply that information along with supporting data on contents of other essential elements.

## MATERIALS AND METHODS

Analyses were made on plants or plant parts taken either from growers' fields or from replicated field experiments designed to evaluate the efficiency of fertility treatments on Red Mexican beans. All samples were dried in a forced-draft oven at 70°C before analysis. These samples were ground in an intermediate Wiley mill with all steel parts to avoid Zn and Cu contamination.

All analyses, except for N, were made on nitric-perchloric acid digests of plant material. Precautions were taken against Zn and Cu contamination. Digests were filtered through paper which had previously been washed twice with hot 1 *N* HCl. The digests were then transferred quantitatively with 1 *N* HCl. Zinc and Cu were determined by the dithizone procedure of Holmes (2) using a Beckman DU spectrophotometer. Calcium and K were determined with a flame photometer, Mg was determined colorimetrically with thiazole yellow, and P was determined with a vanadomolybdate procedure. Total N was determined by the Kjeldahl method using salicylic acid to include nitrates.

[1] Received April 20, 1953.

[2] Scientific Paper No. 1186, Washington Agricultural Experiment Stations, Pullman, Washington.

## PROCEDURE AND RESULTS

ZINC CONTENTS OF PLANT PARTS IN RELATION TO ZINC SUPPLY: Bean plants in early bloom and with four different levels of zinc nutrition were separated into roots, stems, senescent leaves, mature fully-expanded leaves showing no evidence of aging, and growing parts which included very young stems, leaf and flower buds, and immature leaves. The flowers and very small pods were discarded. The root samples were excavated with a shovel and therefore included few of the feeder roots. The roots were washed free of soil with water, then with 0.1 *N* HCl, and finally rinsed several times in deionized water.

The four levels of Zn nutrition varied from very low for plants showing extreme deficiency symptoms to a high level attained by applying $ZnSO_4$ with nitrogen fertilizer in a band near the seed at planting. Plant weights and total Zn contents are shown in table I. Zinc concentrations in the various plant parts are shown in figure 2. The samples for "most severe" and "severe" deficiency consisted of ten plants each collected July 19, 1951, from growers' fields. The samples for "medium" and "high" Zn status, respectively,

FIG. 1. Typical symptoms of zinc deficiency on Red Mexican beans in the field. Scale in inches.

consisted of ten plants from each of three replications of a field experiment.

The "medium" samples were from check plots which yielded 1954 pounds of dry beans per acre.[3] The "high" samples were from treatments receiving 11.6 pounds Zn per acre as the sulfate, placed in a band with the nitrogen fertilizer. This treatment yielded 2423 pounds dry beans per acre, a statistically significant increase of 469 pounds over the non-zinc treatment. Both treatments received 120 pounds of N ($NH_4NO_3$) per acre placed near the seed in a band at planting.

The difference in observed or inferred Zn status was associated with both plant weight and total Zn per plant, as shown in table I. Figure 2 shows that the Zn concentrations in the total tops and the various plant parts also paralleled the assumed Zn status. Maximum differences in concentration were shown in young tissue, followed by the senescent leaves. All plant parts except roots show differences of large magnitude, however. Senescent leaves, mature leaves, stems, and the whole top are of comparable Zn concentration; and each of these categories reflects the Zn status of the plant.

TABLE I

PERTINENT INFORMATION ON BEAN PLANTS SAMPLED AT BLOOM STAGE FROM FIELDS OR EXPERIMENTAL PLOTS, AND USED FOR ZINC DISTRIBUTION STUDIES SHOWN IN FIGURE 2

| | ZINC STATUS | | | |
|---|---|---|---|---|
| | VERY LOW | LOW | MEDIUM | HIGH |
| Deficiency symptoms ..... | Most severe | Severe | None | None |
| Av dry wt per plant (gm) .... | 2.7 | 5.7 | 13.6 | 15.7 |
| Total Zn per plant (mg) ..... | 0.025 | 0.061 | 0.251 | 0.544 |

Leaves showing typical interveinal chlorosis were separated into two fractions: (a) small veins and mesophyll, and (b) midribs and petioles. The Zn contents were 12.8 and 11.4 ppm, respectively. The corresponding Cu contents were 15.8 and 8.0 ppm.

ZINC CONTENT AND DEFICIENCY SYMPTOMS: The relationships between zinc contents of mature leaves or total tops and the presence or absence of definite Zn-deficiency symptoms are shown in figure 3. This relationship is based on samples collected during 1950 and 1951, of plants which varied in size from those about four inches high to those in early bloom at the time of sampling. The "mature leaf" and "total top" samples were obtained from different plants and from different locations.

Zinc contents of "tops" and "mature leaves" show about the same maximum for plants showing symptoms. Zinc contents for "tops" of symptomless plants and deficient plants overlap in the range of 15 to 22 ppm. For "leaves" the overlap is between 17 and 23 ppm. It is probable that plants showing symptoms will contain less than 23 ppm Zn in the leaves or tops, and that plants without obvious symptoms may have as little as 15 ppm in these tissues. Lack of coincidence of these figures lies partly in the subjective judgment of whether or not a plant has symptoms.

[3] Pounds/acre × 0.1121 equals grams/meter².

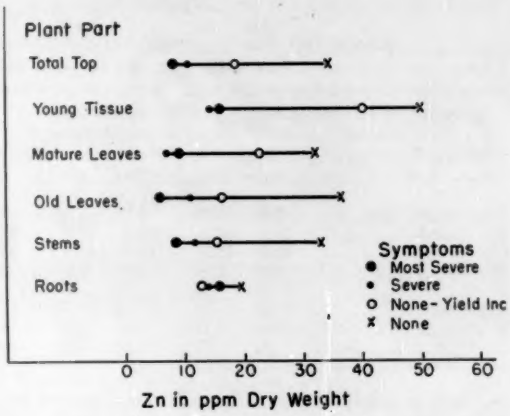


FIG. 2. Zinc contents of parts of bean plants ranging from those unfertilized with Zn and having severe deficiency symptoms to those fertilized with Zn and without symptoms.

EFFECT OF Zn FERTILIZER ON UPTAKE OF Zn AND OTHER ELEMENTS: The average dry weights and composition of plant parts referred to previously in the field experiment where beans were grown with and without $ZnSO_4$ fertilization at planting are shown in table II. These are the "medium" and "high" Zn-level plants of table I and figure 2. All values are means of three replications. Total nutrients per acre are calculated on the basis of 87,000 plants per acre.

The average plant weight was higher for the Zn treatment than for the control, but the difference was not statistically significant. The soil application of Zn increased the Zn concentration in all plant parts significantly and more than doubled the total Zn in the plant. The increase in Zn content of the tops from Zn fertilization was 0.293 mg per plant or 0.056 pounds

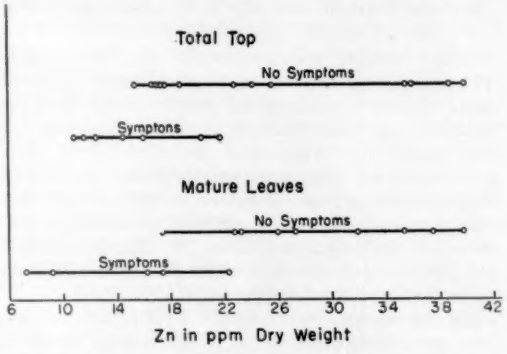


FIG. 3. Zinc content of bean tops and mature leaves in relation to deficiency symptoms.

TABLE II

AVERAGE DRY WEIGHTS PER PLANT AND ANALYSES OF BEAN PLANTS SAMPLED AT BLOOM STAGE WHICH RECEIVED NO Zn ($Zn_0$) AND 11.6 LBS Zn/a. ($Zn_1$) AT PLANTING

| | STEMS | | SENESCENT LEAVES | | MATURE LEAVES | | GROWING PARTS | | TOTAL TOPS | | TOTAL UPTAKE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $Zn_0$ | $Zn_1$ | $Zn_0$ | $Zn_1$ | $Zn_0$ | $Zn_1$ | $Zn_0$ | $Zn_1$ | $Zn_0$ | $Zn_1$ | $Zn_0$ lbs/a. | $Zn_1$ lbs/a. |
| Dry weight (gm) .... | 4.92 | 5.60 | 4.15 | 4.02 | 3.27 | 4.48 | 0.69 | 1.01 | 13.03 | 15.11 | ..... | ..... |
| Zn (ppm) ..... | 15.2 | 32.8 | 16.4 | 36.6 | 22.6 | 32.5 | 40.1 | 50.2 | 18.8 | 34.8 | .048 | .104 |
| Cu (ppm) ..... | 10.9 | 11.2 | 24.1 | 25.8 | 29.1 | 31.7 | 21.7 | 24.4 | 20.2 | 22.2 | .051 | .064 |
| N (%) ..... | 1.94 | 1.66 | 3.14 | 3.09 | 4.40 | 4.52 | 5.33 | 5.46 | 3.12 | 3.14 | 78.0 | 91.0 |
| P (%) ..... | .25 | .25 | .23 | .24 | .36 | .37 | .68 | .69 | 0.29 | 0.31 | 7.3 | 9.0 |
| Mg (%) ..... | .39 | .36 | .52 | .56 | .83 | .79 | .37 | .38 | 0.54 | 0.54 | 14.0 | 16.0 |
| K (%) ..... | 2.93 | 2.74 | 1.89 | 1.91 | 1.69 | 1.65 | 2.53 | 2.55 | 2.27 | 2.18 | 56.7 | 63.2 |
| Ca (%) ..... | .85 | .78 | 1.32 | 1.07 | 2.91 | 2.90 | .53 | .50 | 1.50 | 1.47 | 37.5 | 42.6 |

per acre, a gain of 117%. This additional uptake of Zn amounted to only 0.48% of the 11.6 pounds per acre applied as $ZnSO_4$. Zinc application to the soil and consequent concentration in the plant was accompanied by a slightly increased growth and total absorption of N, P, Mg, Ca, K and Cu, although the concentration of these other elements in the plant was not affected significantly. Copper concentration was highest in the mature leaves, followed by the growing tips and senescent leaves. The distribution pattern was slightly different from that of Zn. Copper and Zn contents were of similar magnitude in these plants.

Concentrations of Ca and Mg were highest in the senescent leaves and lowest in the growing parts. Their distribution is about the reverse of N, Zn and P.

TABLE III

ZINC AND COPPER IN BEAN SEED (OVEN DRY BASIS AT 70°C)

| LOCATION | TREATMENT | YIELD LBS/A. | Cu PPM | Zn PPM |
|---|---|---|---|---|
| Pasco, Washington | None | 19 | 8.0 | 14.0 |
| " " | Zn spray | 1140 | 6.4 | 12.4 |
| Prosser, Washington | None | 1954 | 14.5 | 20.2 |
| " " | 11.6 lbs Zn/a. at planting | 2423 | 13.2 | 19.2 |
| Washington certified seed ..... | ..... | .... | ... | 23.6 |

ZINC AND COPPER CONTENT OF BEAN SEED: Table III gives the Zn and Cu contents of beans produced under as wide a range of Zn supply as the data for vegetative portions shown in figure 2. The samples from Pasco were from check and Zn-sprayed plots where deficiency symptoms were extremely severe and growth was very poor. The samples from Prosser are from the check and $ZnSO_4$ fertilized treatment discussed in connection with table II. The Zn contents in table II are all less than or equal to the minimum values for beans cited by Beeson (1).

The Zn contents shown in table III are of interest from two standpoints. First, if there is an effect of Zn supply on the content in the seed, the effect is much less than that on the vegetative parts as shown in figure 2. The other point of interest is the low Zn content of the seed even when they were produced with Zn fertilization. Even if the Zn in the seed were completely translocated to the top, the latter would show Zn deficiency symptoms by the time its weight equaled that of the seed. As previously mentioned, beans planted on very Zn deficient soils will show symptoms of deficiency before the trifoliate leaves begin to expand.

Washington certified seeds used in the experiment for table II averaged 8.66 μg Zn per seed. The plants produced therefrom without Zn fertilization contained 245 μg per plant at bloom stage. Hence, the seed could have supplied at most only 3.5% of the Zn. These observations and calculations emphasize that a bean plant requires a continuous supply of Zn from time of germination.

## SUMMARY

Zinc deficiency symptoms in field-grown, Red Mexican beans are described. The observation of deficiency symptoms in seedlings soon after emergence is explained by the low Zn content of the seed.

Analysis of plants with different levels of Zn supply, as judged by deficiency symptoms and fertilization experiments, showed that all vegetative parts had a wide range in Zn content. The youngest tissue showed the greatest range.

Deficiency symptoms are associated with about 20 ppm Zn or less in the mature leaves or the total top.

Plants fertilized with 11.6 pounds Zn per acre at planting contained more Zn in all plant parts than did unfertilized plants. Concentrations of Cu, P, K, Mg and Ca were not significantly affected. Substantial increases in dry bean yields were obtained by fertilizing with $ZnSO_4$ even though Zn deficiency symptoms were not apparent on unfertilized plants. Nitrogen, P and Zn showed similar patterns of distribution being highest in young tissues and lower in mature leaves, old leaves and stems. Copper, Mg and Ca were highest in mature leaves.

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# THE INFLUENCE OF COBALT AND SUGARS UPON THE ELONGATION OF ETIOLATED PEA STEM SEGMENTS[1]

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An important function of sugar in cell elongation has been indicated by many authors. For example, Schneider (7) observed that sucrose when externally supplied greatly increased the elongation of Avena coleoptile segments in the presence of indole-3-acetic acid (IAA). Results with etiolated pea stem segments, however, have not been convincing. Galston and Hand (3) reported definite promotion of segment elongation by sucrose but Christiansen and Thimann (1) observed either only slight increases or definite decreases of elongation even though the sucrose moved into the segments and increased the fresh weights. In our laboratory, either promotion or inhibition of elongation of pea stem segments by sugar in the presence of IAA have been observed depending upon the circumstances.

In earlier papers (5, 6) it was shown that cobaltous ions promote the expansion of etiolated bean leaf disks and similarly promote the elongation of etiolated bean stem segments. That cobaltous ions ($3 \times 10^{-4}$ M) when added to a solution of IAA cause a slight increase of the elongation of pea stem segments was confirmed by Thimann (8). The present paper demonstrates that cobalt in the external medium greatly influences the effectiveness of sugar in promoting elongation of pea stem segments in the presence of IAA. The relationship between the action of cobalt and of sugar is considered.

## Methods

Stem segments 5.3 mm long cut from the uppermost portion of the third internodes of 6- to 7-day-old etiolated pea seedlings, var. Alaska #323, were used as test material. The plants were grown in sand in a darkroom maintained at about 25°C and 88% relative humidity and all operations were performed under weak red light. For each treatment, ten segments were floated on 5 ml of the particular solution being tested in a 50-ml Erlenmeyer flask. The initial pH of each solution was 6.0 and solutions were not buffered. The segments were kept in the dark during the growth period of 24 hours.

[1] Received May 11, 1953.

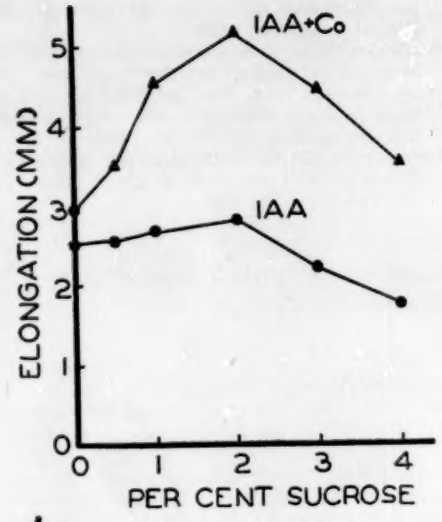
## Results

In preliminary experiments, 1 mg/l IAA was found to be optimal for elongation. Inclusion of sucrose and cobaltous chloride in the solutions did not change this optimal concentration. Therefore, in experiments reported in this paper 1 mg/l IAA was added to all solutions.

In the experiment represented in the lower curve of figure 1, the average increase in length of the segments (originally 5.3 mm long) on the 1 mg/l IAA solution was 2.53 mm. Elongation was greater by 17% on a solution containing the IAA and 2% sucrose, the total increase in length being 2.97 mm. Elongation was inhibited by 3% and even more by 4% sucrose. Slight differences in experimental procedure conceivably could shift the optimum of the curve and perhaps this is why results on the 2% sucrose solutions have been so variable. In eighteen experiments, the growth in length of segments floated on a solution of 2% sucrose and 1 mg/l IAA ranged from 87 to 125% and averaged 104% of the growth of segments floated on solutions containing only IAA.

On a solution of $8 \times 10^{-5}$ M $CoCl_2$ and 1 mg/l IAA, the elongation was slightly but consistently greater than on a solution of only IAA. In eighteen experiments, growth in the presence of the Co + IAA medium ranged from 102 to 134 and averaged 114% of that which occurred on the plain IAA solution. Cobaltous nitrate was equally as effective as cobaltous chloride in these and subsequent tests.

Remarkable increases of elongation were obtained by the addition of 2% sucrose to solutions containing 1 mg/l IAA plus $8 \times 10^{-5}$ M $CoCl_2$. In twenty-one experiments, growth in length on the IAA + Co + sucrose solution ranged from 144 to 239 and averaged 168% of that which occurred on a solution containing only IAA and sucrose. In the presence of 2% sucrose, the optimal concentration of $CoCl_2$ was $8 \times 10^{-5}$ M (fig. 2). That both cobalt and sugar must be present for this promotion of elongation is evident from inspection of the curves of figure 1; neither the sugar nor the salt has more than a slight promotive effect in the absence of the other.



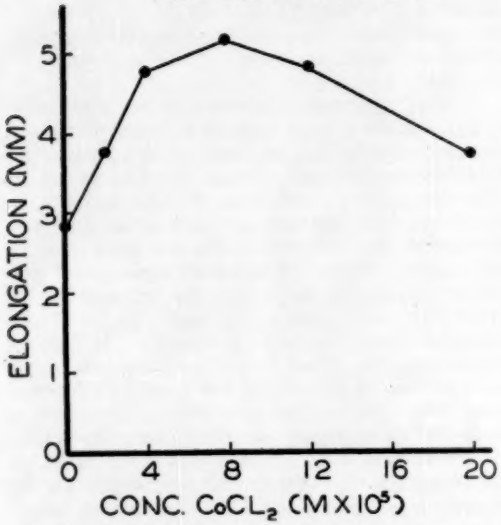




FIG. 1 (*above*). Elongation of etiolated pea stem segments on solutions of various concentrations of sucrose with and without $CoCl_2$ ($8 \times 10^{-5}$ M). All solutions contained 1 mg/l IAA. Orig. length = 5.3 mm.

FIG. 2 (*below*). Effect of various concentrations of $CoCl_2$ upon elongation of etiolated pea stem segments floated on solutions of 1 mg/l IAA and 2% sucrose. Orig. length = 5.3 mm.

Other sugars may be substituted for sucrose. Both fructose and D-glucose apparently are about as effective as sucrose in promoting elongation in the presence of cobalt (table I). The two hexoses reduced elongation when added to test solutions containing only IAA but increased growth when added to IAA + Co solutions.

On the assumption that cobalt might increase elongation by promoting the transformation and utilization of the sugar, the following chemicals were tested

TABLE I

EFFECT OF SUGARS AND $CoCl_2$ ($8 \times 10^{-5}$ M) ON ELONGATION OF ETIOLATED PEA STEM SEGMENTS. ALL SOLUTIONS CONTAINED 1 MG/L INDOLE-3-ACETIC ACID. ORIG. LENGTH = 5.3 MM

| SUGAR | ELONGATION (MM) | |
|---|---|---|
| | WITHOUT $CoCl_2$ | WITH $CoCl_2$ |
| ——— ..... | 2.74 | 3.13 |
| Sucrose (2%) ..... | 2.39 | 5.69 |
| D-Glucose (1%) ..... | 2.35 | 4.57 |
| Fructose (1%) ..... | 2.26 | 5.11 |

in combination with 1 mg/l IAA: glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, phosphoglyceric acid, pyruvic acid, malic acid, succinic acid, galacturonic acid, raffinose, lactose, maltose, galactose, sorbose, arabinose, D-ribose and xylose. None of these gave an increase in elongation.

To test the possibility that cobalt might promote growth by influencing the entry of sugar into the pea stem segments, dry weights of the segments were determined at various times during the period of growth (fig. 3). Dry weights of segments floated on the IAA + sucrose solution increased whereas dry weights of segments floated on the plain IAA solution decreased. Obviously, therefore, some sucrose moved into the segments but cobalt affected neither the time-course of dry weight changes nor the final dry weights.

In all experiments, fresh weights of the segments were increased by inclusion of sucrose in solutions of IAA (1). The swelling of the segments caused by sucrose was great enough to be detected merely by visual inspection. Such increases of fresh weight occurred even when the sucrose did not increase elongation. Inclusion of cobaltous chloride in plain IAA

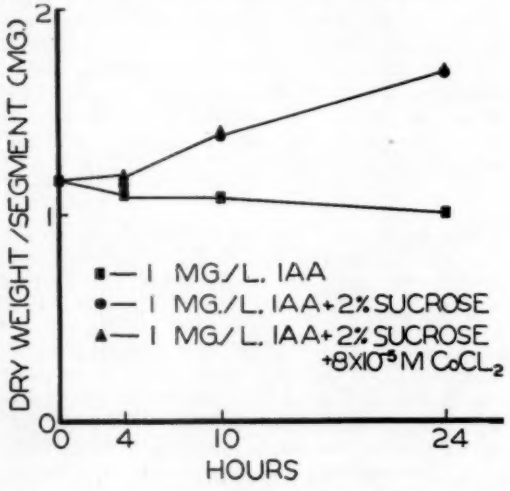



FIG. 3. Dry weights at various times of etiolated pea stem segments floated on test solutions.

solutions did not appreciably affect the fresh weights. On the other hand, inclusion of cobaltous chloride in an IAA + sucrose solution always resulted in increases of fresh weight as measured at the end of the twenty-four-hour growth period. It is an important fact, however, that in some experiments this increase of fresh weight due to cobalt occurred mainly after a cobalt stimulation of elongation was observed. In table II are listed the increases of length and fresh weight in stem segments during successive periods of one experiment. During the first four hours, there were no great differences between any two treatments. During the 4–10-hour period, the increase of fresh weight on the IAA + sucrose solution was approximately double that which occurred on the plain IAA solution even though the increases of length were the same. During this same period, the cobalt chloride

TABLE II

INCREASE IN LENGTH AND FRESH WEIGHT OF ETIOLATED PEA STEM SEGMENTS DURING SUCCESSIVE PERIODS OF GROWTH. ORIG. LENGTH = 5.3 MM. ORIG. FRESH WT. = 0.0130 GM.

| SUBSTRATE * | PERIOD (HR) | Δ LENGTH (MM) | Δ FRESH WEIGHT (GM) | Δ LENGTH (MM) / Δ WEIGHT (GM) |
|---|---|---|---|---|
| IAA | 0–4 | 1.84 | 0.0080 | 230 |
| | 4–10 | 0.95 | 0.0034 | 279 |
| | 10–24 | 0.29 | 0.0016 | 181 |
| | 0–24 | 3.08 | 0.0130 | 237 |
| IAA + S | 0–4 | 1.99 | 0.0072 | 276 |
| | 4–10 | 0.96 | 0.0070 | 137 |
| | 10–24 | 0.41 | 0.0047 | 87 |
| | 0–24 | 3.36 | 0.0189 | 178 |
| IAA + S + Co | 0–4 | 2.04 | 0.0078 | 262 |
| | 4–10 | 1.43 | 0.0075 | 191 |
| | 10–24 | 1.47 | 0.0085 | 173 |
| | 0–24 | 4.94 | 0.0238 | 208 |

* IAA = 1 mg/l indole-3-acetic acid; S = 2% sucrose; Co = $8 \times 10^{-5}$ M $CoCl_2$.

in the IAA + sucrose solution did not affect much the increase of fresh weight but did cause a considerable increase of elongation. These relationships are made quite evident by the ratios presented in table II. Sucrose caused a rapid drop in the ratio of elongation to increase of fresh weight. Cobalt to a great extent prevented this drop by primarily increasing segment elongation. The increases of length and of fresh weight (increase of segment volume) apparently may occur independently and are limited by different factors after the initial increases have occurred. Sugar promotes increases of volume and cobalt primarily promotes elongation.

The data of table II may be considered in terms of decay of the growth rates. On a plain IAA solution, both the rates of elongation and of fresh weight increase fall off quickly. Sucrose slows the decay of the rate of fresh weight increase considerably more than it slows the decay of the elongation rate. The combination of sucrose + cobalt markedly retards decay of both rates. That the decay of the growth rates is not caused by the using up of IAA has been indicated by experiments in which segments were transferred to fresh IAA + sucrose solutions at various times after the start of the tests; this treatment did not increase elongation.

## DISCUSSION

It may be concluded from data presented that increases of cell length and of cell volume in etiolated pea stem segments may be limited differentially by various factors. Sugar consistently causes an increase of cell volume but its effect on elongation is variable. One interpretation of this is that the effect of sugar on elongation depends upon the ability of the cell wall to increase in surface. If other factors are restricting the increase of cell wall surface, the sugar, by increasing the cell volume, causes a rounding-out of the cells of the segments and thereby causes an inhibition of elongation. If these other factors are less limiting, an increase of cell volume brings about an increase of cell length as well.

In line with this interpretation, it is suggested that cobalt in some manner increases the ability of the cell wall to expand. This results in large increases of cell length only when the cell volume is being increased. Thus, the combination of the cobaltous salt and sugar greatly augment elongation of pea stem segments by affecting increases in cell wall surface and cell volume respectively. Of course, it is to be expected that sugar also is involved in the synthesis of cell wall components. The elongation of Avena coleoptile segments, in contrast to pea stem segments, is greatly increased by sucrose (7) but on the other hand the elongation is not promoted by cobaltous ions (8). Here, it is suggested that the coleoptile and pea stem segments differ in the ability of their cell walls to increase in surface area and the condition which cobalt affects is not limiting to elongation in the coleoptile sections.

The mechanism of the cobalt effect is not yet known. However, it is probably the same in the pea stem segments and in etiolated leaf disks because the concentration curves (fig. 2) (5) are almost identical. It has already been suggested (6) that in leaf disk expansion cobalt and light affect the same limiting condition. If that conclusion and the interpretations presented in the present paper are valid, then the effect of small quantities of light specifically is to increase the ability of leaf cell walls to increase in surface area rather than simply to promote an increase of cell volume (water uptake). Furthermore, if these conclusions are correct, the great variability in response of pea stem segments to sucrose may be due to small differences in light treatment of the intact pea plants. The probability that light treatment of intact pea plants affects growth of excised stem portions has been suggested by Kent and Gortner (4).

A similarity between the cobalt and light effects is also apparent from results obtained in preliminary ex-

periments in which we have confirmed the observation by Galston and Baker (2) that elongation in the dark of green pea stem segments is much more dependent upon sucrose than is the elongation of etiolated stem segments. Furthermore, since we have detected no promotion by cobalt of the growth of the green segments, the condition affected by cobalt apparently is not limiting to elongation. In one experiment, the elongation of such stem segments (originally 5.3 mm long) was 1.52 mm on 10 mg/l IAA, and 4.02 mm on IAA plus 2% sucrose. The elongation on either solution was not increased by inclusion of $CoCl_2$ in the concentration range from 4 to $20 \times 10^{-5}$ M.


## Summary

Sucrose in some experiments slightly promoted and in others slightly inhibited the elongation of sub-apical segments from etiolated pea stems. The sugar, however, markedly increased the fresh weights of the segments. Cobaltous salts, when added to test solutions containing only indole-3-acetic acid (IAA), slightly promoted elongation. When sugar and cobalt were added together to the IAA solution, great increases of elongation occurred. The evidence reveals a distinction between the roles of water uptake and cell wall increase in the enlargement of plant cells.

It is suggested that sucrose increases cell volume and that cobalt promotes the ability of the cell walls to increase in surface area.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# APPLICABILITY OF POISEUILLE'S LAW TO EXUDATION OF OLEORESIN FROM WOUNDS ON SLASH PINE[1, 2]

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Yields of oleoresin from wounds on slash pine (*Pinus elliottii* Engelm. var. *elliottii*) and of longleaf pine (*P. palustris* Mill.) are known to vary greatly among trees which are morphologically similar, as shown by Cary (3) and Wyman (16). An estimated 0.5% of the population consists of individuals capable of yielding two to three times more oleoresin than the mean yield for the population, according to Downs (5). Inheritance of capacity to produce high yields of oleoresin has been demonstrated in longleaf pine by Mergen (10), and breeding studies have been started at this station by Dorman (4) and Downs (5) to determine the possibilities of breeding strains of slash and longleaf pine having higher yielding capacities than the average of the existing population. High oleoresin yielding ability is probably attributable to several factors which, if measurable, could be used for selecting individual trees for crossing. The identification of such measurable factors is the purpose of this study.

Many characteristics of pine trees have been measured or suggested for possible correlation with the capacity of the tree to yield oleoresin. These characteristics are listed in the following classification.

A. Morphological factors
   1. crown size (16)
   2. diameter of the bole (16)
   3. radial growth rate of the bole (16)

B. Anatomical factors
   1. size of tracheids (11)
   2. number of longitudinal resin ducts (8, 11)
   3. diameter of longitudinal resin ducts (11)

C. Physical and chemical factors
   1. turpentine content of oleoresin (2, 13)

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[2] A preliminary report on the results of this work was presented at the annual meeting of the Plant Physiology Section, Association of Southern Agricultural Workers, February 4–6, 1952, in Atlanta, Georgia.

2. osmotic pressure of epithelial cells surrounding resin ducts (11)
3. rate of absorption of water by epithelial cells (8, 11)
4. frictional resistance to flow on walls of ducts (8)
5. viscosity of oleoresin (8, 13)
6. pressure on oleoresin in ducts (1, 8, 14)

Correlation between yields of oleoresin and the morphological factors listed above have been observed by Wyman (16), but the degree of correlation is not great. Unpublished data from a recent study at this station show that diameter and radial growth rate during several years preceding oleoresin extraction accounted for about 40% of the variation in oleoresin yields from samples of slash and longleaf pine. Hence these morphological criteria are not very reliable for selecting trees having high productive capacity for oleoresin.

For the anatomical and physico-chemical factors, quantitative data are not sufficient to determine the degree of correlation with oleoresin yielding capacity of individual trees. All of these factors, however, have some conceivable effect on oleoresin yields.

A logical selection of factors which may provide a reliable index of yielding capacity is possible by setting up two hypotheses: 1. The flow rate of oleoresin from a wound during a short time interval may be controlled by the same factors that determine the rate of flow of a liquid through a capillary tube in vitro. 2. The flow rate of oleoresin from a tree during a short time interval may be correlated with the yielding capacity of the tree. If these two hypotheses can be proved, the purpose of the study will be accomplished.

The flow of a viscous liquid through capillary tubes is quantitatively defined by the equation for Poiseuille's Law as modified by Stokes (15) and Hagenbach (7), which is cited by Gortner (6). For true solutions, this equation is

$$(1) \qquad \frac{V}{t} = \frac{\pi r^4 P_0}{8 L \eta}$$

where V = volume of flow
t = time of flow
r = radius of capillary
$P_0$ = hydrostatic pressure on liquid entering the capillary
L = length of capillary
$\eta$ = viscosity of solution

Modifications of this equation are necessary for applying it to exudation of oleoresin. A pressure $P$, was used which differs from $P_0$ above in that $P$ was measured at the point of discharge from the capillary resin ducts. Since $P$ accounts for the reduction in $P_0$ caused by the length $L$ of the capillary, $L$ can be omitted from the equation. This omission may cause a change in the constant, 8. Only relative values for the predicted flow rate are needed for accomplishing the purpose of this experiment, so a constant $K$ can be substituted for $\pi/8$. The interconnected system of longitudinal and radial resin ducts has multiple discharge orifices on any wound surface, hence a factor $N$ for number of exposed radial resin ducts per unit area of tangential wound surface is added to the equation. The transverse sections of the radial resin ducts observed in this study were more elliptical than circular, hence instead of $r$, the major and minor semi-axes of an ellipse, $a$ and $b$ are used. With these modifications the equation becomes

$$(2) \qquad \frac{V}{t} = \frac{KP\sum^{N}(ab)^2}{\eta} \text{ or } Y = \frac{KPN\overline{(ab)^2}}{\eta}$$

where $Y$ is the yield of oleoresin during the first 24 hours after the wounding and $\overline{(ab)^2}$ is the mean of the measured ducts. The applicability of this modified equation was tested.

## Experimental Procedure

The 12 slash pine trees used in this test were growing on a low site with the water table at the surface of the ground at the time of the experiment. The site is in the Olustee Experimental Forest in Baker County, Florida. The trees were marked at random for the test with the restriction that they be at least 25 cm in diameter at breast height. The range in diameter was from 28 to 40 cm. Duplicate sets of observations were made on each of the 12 trees.

Flow Rate of Oleoresin: The flow of oleoresin was measured on August 10, 1951. A steel punch was used to make wounds 37 mm in diameter extending through the bark to the xylem. Wounds were sprayed with a 50% solution of sulfuric acid by weight to open the radial resin ducts which are normally closed at the surface of the xylem. Oleoresin was collected in flat-bottomed glass test tubes suspended on circular plastic fittings, as shown in figure 1 B. The diameter of these fittings is the same as that of a steel punch used in making the wounds. Hence when the fitting is inserted into the hole through the bark, a tight fit is obtained.

Measurements of flow were started after a few drops of oleoresin had drained into the test tube so as to allow time for the level of liquid in the plastic fitting to come to equilibrium. Yields were measured gravimetrically to $\pm$ 0.01 gm. Although in the equation tested, the yield unit is volumetric, substitution of a gravimetric unit introduces a negligible error because the specific gravity of oleoresin is only slightly greater than 1.00. Yields were measured for about 24 hours and the time of flow for each sample recorded to the nearest minute. Flow rates were then computed as gm/hr.

Viscosity: A major difficulty in determining viscosity of oleoresin is to get samples before the resin acids crystallize out of solution. To overcome this difficulty, samples were collected in a closed system which minimized loss of the solvent (turpentine), and the viscosity determinations were made on the day of collection. In this experiment, samples were obtained at the beginning of the 24-hour period of flow measurements, from the same wounds on which flow rates were determined. Samples weighing about 2 gm were

collected in small viscometer tubes (fig. 1 A) each of which had been previously calibrated against a standard. Collection time for viscosity samples was between one and two hours for most trees, but on one, 20 hours were required.

Viscosity was determined by the bubble rise method which involves the time required for an air bubble to rise through the liquid in a vertically mounted tube. The small tubes used in this test were calibrated against a standard Gardner viscometer tube in which the time of bubble rise is said to equal the viscosity in Stokes. Calibration of the standard tube for accuracy of absolute units is not necessary for relative values and was not done. All samples were run in a water bath at 30° ± 0.02°C. The time required for bubble rise was repeated on each sample until three successive observations were obtained which checked within 2%.

Pressure: Closed-end manometers were used for measuring pressure of gum at the point of discharge from the resin ducts. The manometers were made

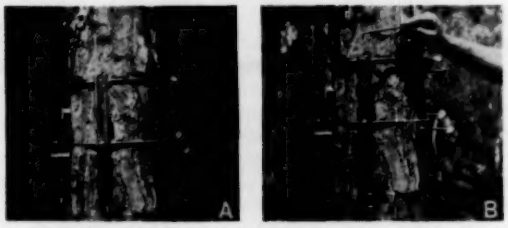


Fig. 1. (A) Installation on tree for measuring exudation pressure of oleoresin with a closed-end manometer clamped against a cambium-depth wound (above) and the receptacle for collecting samples of oleoresin for viscosity determinations. (B) Viscometer tubes shown in (A) are replaced with larger vials in which oleoresin yields are collected and weighed.

from heavy-walled capillary tubing with an inside diameter of about 2 mm and a length of about 250 mm.

The exact length of the manometers was measured after sealing one end and before making the 90° bend near the open end. A millimeter scale cut from graph paper was glued to the outside of the tube. Tubes were dried at 110°C for 2 hours to remove moisture from the bore. The bores were kept dry in a desiccator until installed on the tree. This precaution was taken to delay the occurrence of crystallization of the oleoresin which takes place rapidly in the presence of water.

With a uniform bore within each manometer, pressure in atmospheres on a liquid entering the open end of the manometer is the total length divided by the length of the unfilled portion of the tube. The accuracy of this ratio was confirmed on two of the manometers using a deadweight gauge tester. The ratio is accurate only with a rising pressure. The film of liquid remaining on the wall of the tube causes error in the ratio when pressure is decreasing.

For setting up the manometers, a smooth flat surface about 10 cm in diameter was prepared on the bark of the tree with the center at the desired point of attachment. Selection of a point of attachment in the center of a large bark plate was essential for a good seal. A circular wound 10 mm in diameter was made in the center of the prepared surface and sprayed with a 50% solution of sulfuric acid. The open end of the manometer was pushed through a number 10 rubber stopper so that about 2 mm of the length extended through the stopper. The open end of the manometer was placed in the wound and held there by pressure obtained with a system of cables, clamps, and stretchers as shown in figure 1.

Although this method of measuring pressure of exuding oleoresin involves very little injury to the tree, it was not satisfactory because as soon as appreciable pressure was built up in the system, loss of pressure occurred by penetration of oleoresin into the exposed xylem and bark. The phenomenon probably prevented the establishment of pressure in the manometer in equilibrium with that in the resin ducts.

In use, the manometers were set up during the afternoon of the day preceding the flow measurements so as to show, as nearly as possible, the equilibrium pressure at the time of making the wounds for flow measurements.

Maximum resin pressure in the diurnal cycle occurred shortly after sunrise (between 5:30 and 6:30 A.M.). This maximum pressure, although including much experimental error, was used in the equation to find out if it contributed anything at all to the flow rate. Subsequent pressure readings were made, but because of the loss of oleoresin from the system by penetration of oleoresin into the xylem and through external leaks which developed in a few of the systems, these readings were useless.

Resin Duct Measurements: The material on which radial resin duct measurements were to be made, was collected in the field with the aid of a bow saw, chisel and mallet. Two parallel saw cuts 2.5 cm apart were made first. The first cut was just above the lowest point of the punch wound. A block of wood measuring $2.5 \times 3.2 \times 2.5$ cm was then taken out with the aid of the chisel and mallet. Immediately after collection, the block was dropped into a bottle containing a formalin-acetic acid-alcohol-water (13–7–40–40) fixing and killing fluid. All samples were left in this reagent at least 24 hours.

Blocks suitable for a sliding microtome were sawed off and trimmed out with a knife. Tangential sections, 20 $\mu$ thick were cut with a sliding microtome equipped with an automatic feed. The wood sample and the knife were kept moist with 50% alcohol by means of a camelshair brush. About three times as many sections as were actually needed were cut, so that the best ones could be picked out for studying after staining and dehydration were completed.

The freshly cut material was transferred to a dish containing acetone for 5 minutes to dissolve the oleoresin. The sections were first stained in Ehrlich's haematoxylin and later counterstained with safranin using methods described by Johansen (9). The stained sections were trimmed off carefully with a microtome

knife so that their respective sides were parallel. Resin duct counts were made under 100× magnification. A complete count was made of the radial ducts within each section, starting at the lower left corner and moving across to the right using the diameter of the field of vision as the width of each strip. The area of each section was determined from measurements made with the mechanical stage.

When examined, the transverse sections through radial resin ducts were more elliptical than circular. Hence the major axis (2a) and the minor axis (2b) were measured including the thickness of the epithelial cells. In a tangential section from each wound, 8 ducts were measured. Flow through any one duct is assumed to be proportional to $(ab)^2$. Hence this value was computed for each measured duct in the section before computing the mean used in the equation. Expressed mathematically, this mean is

$$\overline{(ab)^2} = \frac{\sum^{8}(ab)^2}{8}$$

for each of the two wounds on each of the 12 trees.

Where possible the axes of every fifth duct along each strip were measured but where the fifth duct had ruptured epithelial cells as a result of sectioning, the nearest duct to it was measured. Measurements were made under a magnification of 440× with an ocular micrometer in which each division was equal to 3 $\mu$ at the magnification used.

## Analyses and Discussion of Results

The individual measurements obtained in this study are listed in table I. For regression computations, the sums of the two values on each tree were used.

The first step was to determine the significance of each variable in equation (2). For convenience, this equation was re-written

$$Y = KX_1X_2X_3X_4$$

where $X_1 = 1/\eta$, $X_2 = N$, $X_3 = \overline{(ab)^2}$, and $X_4 = P$. Then the assumption was made that each variable has an unknown power so the equation was rewritten again.

$$Y = KX_1^{b_1}X_2^{b_2}X_3^{b_3}X_4^{b_4}$$

Transforming this equation to logarithms,

$$\log Y = \log K + b_1 \log X_1 + b_2 \log X_2 + b_3 \log X_3 + b_4 \log X_4$$

Tests of significance on each of the coefficients showed that $b_1$ and $b_2$ are significant at the 1% level and that $b_3$ is significant at the 5% level. The coefficient $b_4$ is significant at the 25% level.

Hence the observed values of N, $\overline{(ab)^2}$, and $1/\eta$ are each correlated with flow rate. Pressure measurements contributed nothing to the precision of the regression because of the inadequate techniques used.

Although accurate measurements of exudation pressure of oleoresin were not obtained, the existence of

Table I

Individual Measurements of the Variables

| Tree and wound designation | Flow rate | Viscosity of oleoresin ($\eta$) | Number of ducts per $cm^2$ (N) | $\overline{2a}$ * | $\overline{2b}$ * | $\frac{\overline{(ab)^2}}{10^7}$ | Observed exudation pressure (P) |
|---|---|---|---|---|---|---|---|
| | *gm/hr* | *Stokes* | | $\mu$ | $\mu$ | | *atm.* |
| 1A | 1.792 | 120 | 53.67 | 69.8 | 53.2 | .8838 | 3.95 |
| 1B | 1.619 | 100 | 47.29 | 71.2 | 54.4 | .9715 | 4.40 |
| 2A | 0.324 | 212 | 38.16 | 64.9 | 53.2 | .7861 | 4.22 |
| 2B | 0.156 | 224 | 33.60 | 64.1 | 52.5 | .7543 | 4.24 |
| 3A | 0.619 | 112 | 37.12 | 64.1 | 52.5 | .7311 | 3.88 |
| 3B | 0.500 | 119 | 47.16 | 65.2 | 49.5 | .6861 | 3.96 |
| 4A | 1.094 | 158 | 55.68 | 70.5 | 59.6 | 1.1222 | 3.97 |
| 4B | 1.060 | 157 | 60.45 | 70.9 | 52.1 | .9003 | 3.65 |
| 5A | 0.700 | 155 | 43.57 | 75.4 | 60.0 | 1.3205 | 3.95 |
| 5B | 0.552 | 158 | 47.39 | 73.9 | 54.0 | 1.0166 | 4.24 |
| 6A | 0.869 | 87 | 34.44 | 75.8 | 53.6 | 1.0559 | 4.93 |
| 6B | 0.890 | 78 | 34.23 | 76.5 | 55.5 | 1.1609 | 4.22 |
| 7A | 0.816 | 114 | 56.15 | 67.5 | 54.4 | .8676 | 4.50 |
| 7B | 0.766 | 99 | 45.99 | 62.2 | 52.5 | .6797 | 3.94 |
| 8A | 0.300 | 149 | 33.28 | 74.2 | 52.1 | .9889 | 3.73 |
| 8B | 0.224 | 180 | 39.47 | 63.0 | 51.0 | .6563 | 3.96 |
| 9A | 0.617 | 122 | 26.15 | 72.4 | 59.6 | 1.1964 | 4.51 |
| 9B | 0.754 | 117 | 34.14 | 69.4 | 55.9 | .9676 | 4.57 |
| 10A | 1.004 | 99 | 39.17 | 76.5 | 59.6 | 1.3235 | 3.75 |
| 10B | 0.945 | 112 | 35.45 | 76.1 | 59.6 | 1.3028 | 3.26 |
| 11A | 0.145 | 256 | 36.22 | 73.1 | 54.8 | 1.0166 | 4.34 |
| 11B | 0.141 | 233 | 33.07 | 67.5 | 53.6 | .8535 | 2.99 |
| 12A | 1.305 | 86 | 46.75 | 75.4 | 53.2 | 1.0198 | 5.05 |
| 12B | 1.116 | 104 | 44.98 | 76.9 | 60.4 | 1.4017 | 3.06 |

* $\overline{2a}$, $\overline{2b}$ = Major and minor axes of elliptical transverse sections of radial ducts (mean of 8 ducts).

such pressure was demonstrated. Hence it is probably a determining factor in rate of flow of oleoresin.

To determine how much of the variation in flow rate (in absolute units rather than logarithms) was accounted for by the three significant factors, the following regression equation was set up with the pressure factor omitted:

$$Y = a + bX \qquad \text{where } X = \frac{N\overline{(ab)^2}}{\eta}$$

One solution of this equation is $Y = -0.367 + 2.864X$ This solution is based on the assumption that the variance of Y is proportional to $X^2$.

The deviations of the observed values from the line representing this equation are shown in figure 2. The coefficient of determination is 0.83, which means that the regression accounts for 83% of the variation of Y in the sample of 12 trees. A logical deduction is that some of the remaining 17% of the variance in the

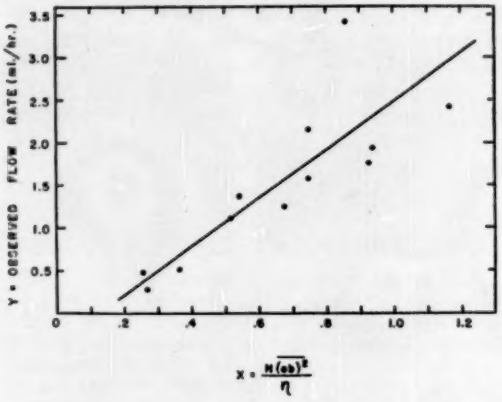


FIG. 2. Deviations from the regression equation

$$Y' = -0.367 + 2.864\,\frac{N\overline{(ab)^2}}{\eta}$$

sample trees could probably have been accounted for with more precise measurements of pressure.

Although the viscosity of gum and the size and number of radial resin ducts are correlated with flow rate during the first 24 hours after wounding, the product of these independent variables can be an index of oleoresin yielding capacity only if flow rate or total yield during the first 24 hours after wounding is correlated with the yielding capacity. The true yielding capacity of a tree is an abstract concept which, at the present time, cannot be quantitatively defined. The actual yield of oleoresin obtained by working a tree for one season with a specified technique is the best available quantitative index of the yielding capacity.

In 1952, individual yield records were made on 25 slash pine trees worked with a standard commercial chipping method. For the wound made on June 26 on these trees the yield during the first 24 hours was determined. The coefficient for the correlation between this one-day yield and the total yield for the season is 0.440, which is significant at the 5% level. Hence the product of the three independent variables is probably correlated with the current annual yield of individual trees.

If the first correlation holds for a stratified sample of high-yielding trees, the probability of getting progeny with oleoresin-yielding capacity greater than that of either parent possibly could be increased by crossing a tree rating high on one factor with another tree rating high with respect to another factor. If all three factors are independently inherited, successive selections and crosses may result in trees rating high on all three factors. Such trees may have an oleoresin-yielding capacity several times greater than that of the existing population.

## SUMMARY

The variables in a modification of the equation for Poiseuille's Law for flow of liquids through capillaries were measured in the resin duct system of 12 slash pine trees (*Pinus elliottii* Engelm. var. *elliottii*). The modified equation is given.

Regression analysis showed that the product of three of the independent variables, i.e., the reciprocal of the viscosity of oleoresin, number and size of resin ducts, accounted for 83% of the variation in flow rate between trees. Pressure could not be measured accurately because of the loss of pressure by penetration of oleoresin into the xylem behind the enclosed wound to which manometers were attached. Hence the improvement in fit of the regression line from the inclusion of pressure values in the product of the independent variables was not significant.

Flow rate of oleoresin during the first 24 hours after wounding was correlated with annual yield of oleoresin which is the only available index of yielding capacity.

The three factors, N, $\overline{(ab)^2}$, and $\eta$, therefore may be useful criteria for selection of parent trees to be used in producing progeny having a high oleoresin yielding capacity.

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# THE EFFECTS OF THREE LEVELS OF MAGNESIUM ON THE NUTRIENT-ELEMENT COMPOSITION OF TWO INBRED LINES OF CORN AND ON THEIR SUSCEPTIBILITY TO HELMINTHOSPORIUM MAYDIS[1, 2]

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This study was undertaken to ascertain the effects of different levels of magnesium on the nutrient-element balance of two inbred lines of corn and its relationship to the incidence of leaf blight caused by *Helminthosporium maydis*. The influence of nutrition on the severity of plant diseases has received considerable attention (3, 7, 15, 16, 17, 18, 20, 21, 22, 23). Wingard (25) gives a general review of the literature pertaining to this subject. In general, the literature supports the view that high concentrations of nitrogen predispose plants to disease, while high potassium tends to retard disease development. Many conflicting cases, however, may be found. Some of the discrepancies may be caused by inadequate knowledge concerning the nutritional status of the plants under study, as reflected by leaf composition, while others may be caused by differences in the nutritional requirements of various pathogens.

Investigations dealing with the effect of magnesium on the susceptibility of plants to disease are limited to a few papers. Bledsoe *et al.* (3) reported a relationship between the magnesium content of leaf tissue and the incidence of peanut leafspot. They found that plants grown at low magnesium levels had less magnesium in the leaf tissue and developed about fifty times more leaf spots than did plants grown at high magnesium levels. A fungus, *Mycosphaerella arachidicola* W. A. Jenk, was isolated from the leaf spots, leading them to believe it was responsible for the disease.

Differences between inbred lines of corn with respect to leaf-blight susceptibility are well established (19, 24). Little is known, however, concerning differences in their nutrient requirements. Using solution culture techniques, Sayre (14) found that growth curves of various inbred lines differed although grown at the same magnesium level. It has been noted from field observations that some inbred lines exhibited visual deficiency symptoms, while others growing nearby did not (10).

Problems involving magnesium deficiency in crop plants can be expected to become more numerous in the future (1). Since little is known regarding the effect of magnesium on disease susceptibility and the nutrition of different inbred lines of corn, it is important that further information be obtained.

## MATERIALS AND METHODS

Two inbred lines of corn, Illinois Hy and Ohio 41, were grown in a greenhouse in nutrient solutions containing three levels of magnesium, while all other elements were kept as constant as possible. A preliminary experiment was carried out to determine the lowest and the highest concentrations which did not produce deficiency or toxicity symptoms respectively. To determine these levels both inbred lines were grown in solutions to which the following amounts of magnesium were added: 0, 3, 5, 10, 25, 45, 60, 75, 100, 250 and 500 ppm. The sole purpose of the preliminary experiment was to study the response of both inbreds to a wide range of magnesium levels and to select the levels most appropriate for further experiments.

This information was used in setting up the main experiment. Three levels of magnesium were chosen: 3 ppm (low), 75 ppm (medium), and 300 ppm (high). The experiment consisted of four replications; magnesium treatments and inbred lines were randomized within each replication. After the plants had developed under the differential magnesium treatments for about 12 weeks, they were inoculated with cultures

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of *Helminthosporium maydis* (Lancaster 2). When the disease incidence reached maximum primary infection, a disease index was calculated. Leaf samples were analyzed for twelve elements to determine the nutrient-element balance of each inbred line.

The nutrient-culture assembly used in these experiments was constructed by Smith (16) for the study of micronutrient elements and their effect on plant disease susceptibility. The containers used to hold the nutrient solutions were four-liter Pyrex beakers. The plants were suspended in the nutrient solutions by wrapping a piece of absorbent cotton around the stalk and inserting them through 2-cm holes drilled in 30-cm square asbestos board covers. The covers were placed on top of the beakers and excluded all light from the solutions. Air was forced through fritted glass discs to insure adequate aeration of the solutions.

A modification of Hoagland's solution No. 2 as described by Hoagland and Arnon was used (9). The Mg was supplied as $MgCl_2$ and S as $Na_2SO_4$. The following concentrations resulted from the use of this formula (ppm); N, 210; P, 31; K, 234; Ca, 160; S, 64; B, 0.5; Mn, 0.5; Cu, 0.02; Zn, 0.22 and Mo, 0.01. Iron was added twice weekly to each beaker at the rate of two ml of 0.5% ferric tartrate. Magnesium was added in the quantities needed by the introduction of suitable aliquots from stock solutions containing 5 gm/l or 50 gm/l Mg. The period between changes of solution averaged 14 days, the frequency depending on the size and development of the plants.

The plants were started in pure quartz sand from seed obtained from the Pennsylvania Foundation Seed Stocks. On June 24, 1950, when the young plants had attained a height of 5 cm they were transferred to the nutrient-culture assembly.

On September 9, 1950, the plants were inoculated with mycelium and spores of *Helminthosporium maydis*. Preliminary tests of several races of this pathogen showed the race Lancaster 2 to be quite virulent with respect to the two inbred lines of corn under study. The inoculum was produced by streaking Petri dishes containing 15 ml of cornstarch agar with mycelium of the pathogen; two weeks later, uniform discs of the mycelium-bearing agar were removed from the dishes by means of a 3-mm cork borer. To eliminate any differences between cultures the discs were randomized before being placed on the plants. One disc was gently pressed upon the fifth and sixth leaves, counted from the base, of each plant at a point midway between the midrib and leaf margin and about 15 cm from the tip. The inoculations were made in the evening so that infection would take place when the greenhouse was cool.

A clear plastic parafilm tent was placed over the entire nutrient-culture assembly and a humidifier was operated under it to furnish a high humidity favorable for the development of the pathogen.

The index of disease incidence was taken on October 4, 1950, when the disease lesions had developed to a maximum and secondary infection had commenced. The inoculated leaves were removed from the plants and the area of the disease lesions was measured by means of a planimeter. Each lesion was measured twice and the mean of the two readings was recorded in $cm^2$. Only those lesions which directly surrounded the original point of inoculation were measured; no attempt was made to measure secondary infection.

Leaf sampling was carried out on September 19, 1950, with the removal of the seventh, eighth, and ninth leaves from each plant. They were wiped with damp cheesecloth to remove contaminating substances, dried in an oven at 60°C, and ground in a Wiley mill through a 40-mesh screen.

The minerals, P, K, Ca, Mg, Na, Fe, Mn, Cu and Zn were determined by the leaf analysis methods of Parks *et al.* (12) as modified by Smith (16). Total N was determined on a separate 0.5-gm sample using the Winkler modification of the Kjeldahl-Gunning method (13). A second 0.5-gm sample was used for the determination of boron by the method of Austin and McHargue (2).

All results reported are the means of closely agreeing duplicates except in a limited number of cases where sample size restricted the analysis to a single determination. The leaf-analysis data were subjected to analysis of variance as described by Goulden (8), while the disease data was treated statistically by means of the "t" test.

## Experimental Results

The nutrient-element balance of both inbred lines was significantly altered as a result of varying Mg treatments. It will be noted from table I that there was a consistent increase in leaf concentration of Mg when this element was increased in the nutrient medium from a low level (3 ppm) to the medium

### Table I

The Effects of Three Levels of Magnesium on the Nutrient-Element Composition of Two Inbred Lines of Corn

| Per cent dry weight | Magnesium level | | | | | |
|---|---|---|---|---|---|---|
| | 3 ppm | | 75 ppm | | 300 ppm | |
| | Ill. Hy | Ohio 41 | Ill. Hy | Ohio 41 | Ill. Hy | Ohio 41 |
| N | 3.05 * | 2.91 | 2.91 | 2.72 | 2.66 | 2.55 |
| P | .54 | .37 | .53 | .31 | .46 | .30 |
| K | 2.80 | 2.82 | 2.57 | 2.08 | 2.04 | 1.97 |
| Ca | .78 | .61 | .72 | .76 | .63 | .56 |
| Mg | .11 | .10 | .36 | .44 | .58 | .66 |
| $SO_4$ | .42 | .40 | .36 | .34 | .24 | .29 |
| | μg per gm dry weight | | | | | |
| Na | 154 | 108 | 120 | 135 | 135 | 136 |
| Fe | 120 | 122 | 135 | 143 | 135 | 126 |
| Mn | 52 | 60 | 60 | 50 | 49 | 50 |
| Cu | 35 | 31 | 41 | 29 | 32 | 28 |
| Zn | 41 | 41 | 59 | 33 | 46 | 31 |
| B | 27 | 42 | 32 | 25 | 36 | 34 |

* Each value represents the mean of four replications.

TABLE II

THE EFFECTS OF MAGNESIUM ON THE MEAN NUTRIENT-ELEMENT COMPOSITION OF TWO INBRED LINES OF CORN

| Mg LEVEL PPM | PER CENT DRY WEIGHT | | | | | |
|---|---|---|---|---|---|---|
| | N | P | K | Ca | Mg | $SO_4$ |
| 3 | 2.98 * | .45 | 2.81 | .70 | .10 | .41 |
| 75 | 2.82 | .42 | 2.33 | .74 | .40 | .35 |
| 300 | 2.60 | .38 | 2.00 | .60 | .62 | .27 |
| LSD 19 : 1 | .15 | .04 | .23 | .04 | .05 | .06 |
| LSD 99 : 1 | .21 | .06 | .32 | .06 | .07 | .09 |

| Mg LEVEL PPM | MICROGRAMS PER GRAM DRY WEIGHT | | | | | |
|---|---|---|---|---|---|---|
| | Na | Fe | Mn | Cu | Zn | B |
| 3 | 131 | 121 | 56 | 33 | 41 | 34 |
| 75 | 128 | 139 | 55 | 35 | 46 | 28 |
| 300 | 136 | 130 | 50 | 30 | 38 | 35 |
| LSD 19 : 1 | N.S. | N.S. | N.S. | N.S. | N.S. | N.S. |
| LSD 99 : 1 | ... | ... | .. | .. | .. | .. |

* Means of Illinois Hy and Ohio 41, each replicated four times.

level (75 ppm) and to the high level (300 ppm). In the case of Ohio 41 this increase was nearly seven-fold, while in that of Illinois Hy it was over five-fold. Uptake of N, P, K, and S was significantly depressed as a result of higher Mg levels. The effects of the three Mg levels on the mean nutrient-element composition of the two inbred lines and the differences required for statistical significance as computed by an analysis of variance may be seen in table II.

Under the same environmental conditions the two inbred lines absorbed significantly different quantities of some nutritionally important elements. It will be observed from table III that Illinois Hy absorbed more N, P, Ca, Cu and Zn; while Ohio 41 absorbed more Mg.

TABLE III

COMPARISON OF THE MEAN NUTRIENT-ELEMENT COMPOSITION OF THE INBRED LINES ILLINOIS HY AND OHIO 41

| INBRED LINE | PER CENT DRY WEIGHT | | | | | |
|---|---|---|---|---|---|---|
| | N | P | K | Ca | Mg | $SO_4$ |
| Illinois Hy .. | 2.87 * | .51 | 2.47 | .71 | .35 | .34 |
| Ohio 41 ..... | 2.73 | .33 | 2.29 | .64 | .40 | .34 |
| LSD 19 : 1 ... | .12 | .03 | N.S. | .04 | .04 | N.S. |
| LSD 99 : 1 ... | N.S. | .05 | N.S. | .05 | N.S. | .. |

| INBRED LINE | MICROGRAMS PER GRAM DRY WEIGHT | | | | | |
|---|---|---|---|---|---|---|
| | Na | Fe | Mn | Cu | Zn | B |
| Illinois Hy .. | 136 | 130 | 54 | 36 | 49 | 32 |
| Ohio 41 ..... | 126 | 130 | 53 | 29 | 35 | 34 |
| LSD 19 : 1 ... | N.S. | N.S. | N.S. | 4 | 2 | N.S. |
| LSD 99 : 1 ... | ... | ... | .. | 5 | 3 | .. |

* Means of four replications and three magnesium treatments.

The disease susceptibility of both inbred lines was altered as a result of changes in the intensity and balance of nutrition brought about by the three magnesium levels in the nutrient solutions. Table IV shows the incidence of disease for both inbreds at the three Mg levels with the accompanying statistical significance. As the magnesium concentration of the nutrient solutions was increased there was a consistent increase in disease incidence of both inbred lines. With the inbred line Ohio 41 there was a highly significant increase in size of disease lesions on plants grown at the medium magnesium level when compared with those on the plants grown at the low magnesium level. A like significance was obtained when comparison was made between the low and the high levels. There was no significant difference between the medium and high levels even though disease incidence was greater at the high level.

Illinois Hy exhibited the same trend in disease susceptibility as did Ohio 41. The differences while consistent were not so great and were not statistically

TABLE IV

THE EFFECTS OF THREE LEVELS OF MAGNESIUM ON THE INCIDENCE OF DISEASE IN TWO INBRED LINES OF CORN

| INBRED LINE | DISEASE INDEX ($CM^2$) AT THREE MAGNESIUM LEVELS (PPM) | | | DIFFERENCES BETWEEN Mg LEVELS | | |
|---|---|---|---|---|---|---|
| | 3 | 75 | 300 | 3×75 | 75×300 | 3×300 |
| Ohio 41 .... | .97 * | 1.36 | 1.70 | .39 † | .34 | .73 † |
| Illinois Hy .. | 1.59 | 1.81 | 1.86 | .22 | .05 | .27 |
| Differences between inbreds ... | .62 † | .45 † | .16 | .. | .. | .. |

* Means of four replications.
† Significant at the 1% point.

significant. There was a marked increase in disease incidence at the medium Mg level when compared with the low level. A higher concentration of Mg in the nutrient medium failed to bring about an appreciable increase in disease incidence.

A comparison of the disease susceptibility between the two inbred lines shows Illinois Hy to be more susceptible to *Helminthosporium maydis* at all the magnesium levels used in this experiment. At the low and medium levels the differences are highly significant, while at the high level no significant difference exists.

## DISCUSSION

Both inbred lines exhibited similar trends with respect to disease susceptibility at the three Mg levels. Disease incidence of the inbred line Illinois Hy was not increased by larger increments of magnesium to the same extent as it was in the other inbred line. This is especially true of the high magnesium level. The failure to obtain greater disease incidence at the high Mg level is in part based on the growth char-

acteristics of this inbred line. Throughout these experiments the growth of Illinois Hy was characterized by narrow leaves as contrasted to the broad leaf of Ohio 41. When disease measurements were taken it was noted that at the high level many of the disease lesions on Illinois Hy had spread from the point of inoculation to the leaf margin and were thus unable to develop further in a lateral direction. This undoubtedly resulted in lower readings than would have been obtained had the lesions been able to develop freely. Thus disease susceptibility of Illinois Hy at the high Mg level was conditioned in part by a factor other than nutrition of the suscept.

Under the conditions of this experiment disease susceptibility of the inbred lines was influenced to a varying degree by the nutrition of the suscepts, and their nutrition in turn was significantly altered by the

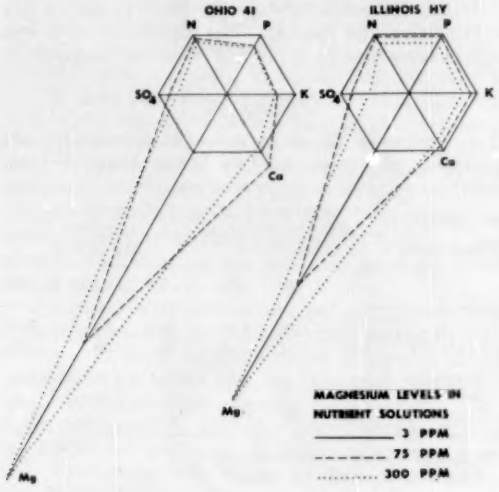


FIG. 1. The effects of three levels of magnesium on the leaf composition of Ohio 41 and Illinois Hy.

Mg level under which they were grown. Leaf analysis revealed the extent and direction of changes in nutrition resulting from the three magnesium levels. On the basis of the leaf-analysis data it would appear that the relationship between nutrition and disease susceptibility cannot be traced to the effect of a single element. While the Mg content of the leaf tissue increased many fold at the same time that disease incidence increased, there was also a consistent decrease in the concentration of N, P, K, and S. Thus the relationship between disease incidence and nutrition appears to be related to the quantitative effects of these five elements.

Figure 1 is designed to show the relationship between these five elements together with calcium and the incidence of disease. The construction of the figure is based on the method of Nicholas (11) and is drawn by plotting the analytical results of the leaf analyses from the magnesium treatment resulting in the lowest disease incidence, in this case the low level, on the radii so that a regular polygon is formed when the vertices are connected. All other treatments are superimposed on the regular polygon using the scale already established. Thus any increase or decrease in the concentration of any one of the elements results in a change in the shape of the polygon. It will be noted from figure 1 that as disease incidence increased there was a consistent decrease in the concentration of N, P, K, and S; while Mg showed a large increase. However, there was also a change in the ratio or balance of nutrition with each successive increment of magnesium applied in the nutrient solutions. Calcium, which failed to exhibit a consistent relationship to disease incidence in the inbred line Ohio 41, has been included in the diagram because of its importance in relation to the other bases.

It is interesting to note the similarity of results obtained by Thomas and Mack (18) using the method of foliar diagnosis in a study of tomato wilt caused by *Fusarium lycopersici* Sacc. and those found in this study. They showed that a disequilibrium between N, $H_3PO_4$, and K was the principal cause for variation in disease susceptibility. Furthermore, the intensity of nutrition as expressed by the sum of the percentages of N, $P_2O_5$, and $K_2O$ was lower in leaf samples from plots containing diseased plants than in comparable samples from plots in which all plants were healthy, and decreased as the severity of the disease increased. These findings are in accord with those reported here, in that, as disease incidence increased the nutrient-element balance of the plants was altered with a simultaneous decrease in the intensity of N, P, and K.

Before definite conclusions are drawn regarding the influence of Mg nutrition on the disease susceptibility of corn it would appear advisable to study this problem further under field conditions to determine if the relationship reported here for greenhouse nutrient-culture conditions is also apparent under field conditions. Complete leaf analysis data should be obtained to determine the leaf concentrations at which disease incidence was at a minimum and yield at a maximum. Information concerning the nutritional relationship between suscept and pathogen should prove of value in gaining further insight into the nature of disease susceptibility.

Knowledge concerning differences in the absorption of nutrient elements by various inbred lines when grown under the same environmental conditions may be of importance in selecting lines for hybridization. Such knowledge may help to explain the superiority of one inbred line over another in ability to obtain or utilize nutrients and thus aid in selecting more vigorous lines.

## SUMMARY

Two inbred lines of corn, Illinois Hy and Ohio 41, were grown in a greenhouse in nutrient solutions containing three levels of magnesium. After growing in the nutrient solutions for 11 weeks, the plants were inoculated with cultures of *Helminthosporium maydis*.

When the primary disease lesions had reached a maximum size, a disease index was calculated based on the area of the lesions. Leaf samples were taken from both inbred lines after they had been inoculated and were analyzed chemically for twelve elements to determine the effect of the three magnesium levels on the nutrient-element composition.

The different magnesium levels significantly altered the nutrient-element composition of both inbred lines. Progressive increments of magnesium in the nutrient solutions were reflected in a highly significant increase of this element in the leaf tissue and a significant decrease in the nitrogen, phosphorus, potassium, and sulphur content.

As a result of the modification in the nutrient-element composition, disease susceptibility of the plants was consistently increased from the low magnesium level to the high. In the case of Ohio 41 the difference between the low and medium magnesium levels was statistically significant as was that between the low and high levels. There was no significant difference between the medium and the high levels. While Illinois Hy exhibited the same trend with regard to disease susceptibility, the differences were not statistically significant.

Evidence is presented which indicates that the relationship between increased disease incidence and nutrition of the suscepts was related to the quantitative and qualitative effects of nitrogen, phosphorus, potassium, sulfur, and magnesium.

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# AUXIN RELATIONSHIPS OF THE RICE COLEOPTILE[1]

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It has long been known that the rice coleoptile is not only capable of growth under water but that this organ actually grows more extensively under water than in air. This property of the rice coleoptile has an important practical significance for rice culture. Thus, in the United States, rice seeds are usually sown by airplane in water which is kept 5–6 inches deep over the field. The seeds germinate under water and the coleoptiles grow up to the surface of water within a few days. In the northern part of Japan, the seed bed, after seeding, is frequently flooded relatively deeply in order to prevent cooling of the soil during cold weather. In this case also, the rice coleoptile grows under water.

That the rice seed is capable of germination in an atmosphere of reduced oxygen concentration as is the case under water has been shown directly by Yokoi (21), Takahashi (15), Sasaki (14), Edward (6) and Morinaga (9, 10). In these reports it has also been found that the growth of the rice coleoptile under water is greater than in air. Vlamis and Davis (19) have reported a detailed study of the growth of the rice coleoptile in relation to oxygen tension, and have compared rice with barley in this respect. It was found that rice shoot growth (measured by fresh weight) is at a minimum in 21% oxygen (air) and increases with decreasing oxygen tension, reaching a maximum at an oxygen tension of about 3%. Taylor (17, 18) has studied the influence of oxygen tension on the respiratory rate, fermentation, and growth of rice seedling, and has compared rice with wheat. Other attempts have also been made to correlate the capacity of rice to germinate and grow under water with such physiological characteristics as fermentation, production of alcohol, tolerance to alcohol, or particular changes in enzyme activities of rice when it is grown under water (11, 8, 7, 17, 12). No clear correlations of this kind have, however, been established, and the physiological basis of the peculiar characteristics of the growth of seedling rice have remained obscure.

This paper deals with the auxin relationships of rice coleoptile growth. The elongation of this organ depends, as shown in figure 1, on submersion, being greater when the seedling is immersed in water than when it is allowed to grow in air. It will be shown that the elongation of the rice coleoptile is auxin dependent, just as is the case with the coleoptiles of Avena. Rice differs from Avena, however, in possessing a highly active system for the destruction of auxin, a system whose activity depends greatly on oxygen tension.

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## MATERIALS AND METHODS

The rice seeds used in these experiments were of the variety "Colusa." Seeds were soaked for ten minutes in a 0.5% solution of sodium hypochlorite and were then rinsed thoroughly with distilled water. The seeds which remained floating during this procedure were discarded, and the remainder selected for uniformity in size and appearance. The selected seeds were then allowed to germinate under water in a dark room at 25 to 26°C. Various cultural procedures were used as noted below.

## EXPERIMENTAL RESULTS

The growth of the rice coleoptile was first investigated in relation to decapitation. For these experiments groups of 20 seeds were placed at the surface, at 5 cm below or 10 cm below the surface of water contained in separate beakers. The elongation of the coleoptile was followed in each instance. The results summarized in figure 1 show that vigorous elongation occurs under water, but that elongation is much lower in seedlings growing at the surface. It may be noted that the elongation of the mesocotyl, known to be more sensitive to auxin than the coleoptile in other cereals (20), was also greater under water than in air. The initiation and growth of the roots on the contrary is inhibited under water.

Coleoptiles growing in air (seeds placed on the surface of water) or under water (5 cm depth) were

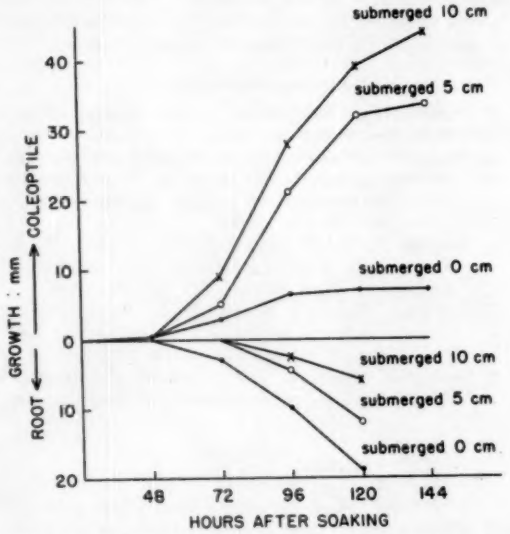


FIG. 1. Growth of rice coleoptiles in air and under water. Seeds placed at 0, 5 and 10 cm below the water surface.

decapitated by removing 2 to 3 mm tips, and after four hours a second decapitation was made. The elongation of the coleoptiles in air decreased to zero after the second decapitation. This behavior then is similar to that known for the Avena coleoptile (20). The coleoptile growing under water does not, however, show any appreciable decrease of elongation even after the second decapitation (fig. 2 a). A more drastic decapitation was therefore carried out in which approximately 10 mm of the upper portion of the coleoptile (total length 22 mm) was removed. After four hours a second 3 mm of the apical stump was again

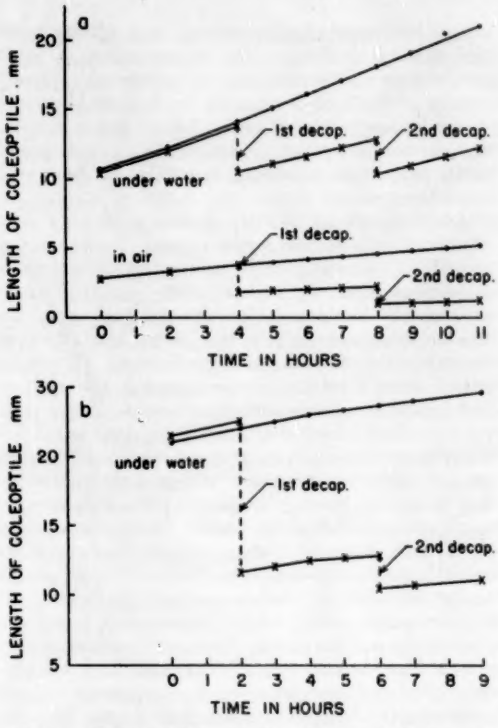


FIG. 2. Effect of decapitation on growth of rice coleoptiles in air and under water. C: control coleoptiles, D: decapitated coleoptiles.

removed. The remaining tissue continued to grow, with no decrease in rate (fig. 2 b). The facts summarized above indicate the possibility that rice coleoptile tissue grown under water may contain such large amounts of auxin, that the removal of the top does not result in any immediate deficiency of auxin in the remaining portion. Coleoptiles grown in air appear on the contrary, to be limited by auxin after decapitation, much as are the coleoptiles of Avena.

It will now be shown directly that the growth of rice coleoptiles does depend on auxin. Seeds were soaked and germinated under water 6 cm in depth and contained in large (24 × 40 cm) trays in a standard Avena dark room. Seedlings with coleoptiles 1–1.5 cm in length were selected, and sections 5 mm long were removed from the portion lying between 3 mm and 8 mm below the top of coleoptile. For this purpose use was made of a special double bladed cutting tool. These sections were placed in lots of 20 in 20 ml of indole acetic acid (IAA) solution of each of a series of concentrations placed in glass vials (2 cm diameter and 8.5 cm high). Each group of 20 sections was either floated on the surface or submerged at about 5 cm below the surface of the solution. This was achieved by use of screen made of nylon net which restrained the sections at the desired depth.

Preliminary experiments showed that the growth response of rice coleoptile sections to IAA decreases with the age of the seedling. As a standard practice, relatively young coleoptiles 1 to 1.5 cm in length were therefore used exclusively. Certain of the early experiments also yielded irregular results owing to the growth of microorganisms in the culture solutions.

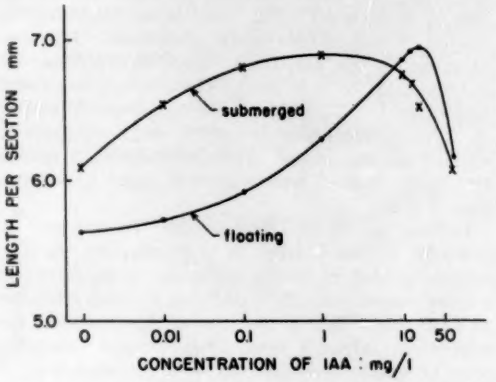


FIG. 3. Growth response of coleoptile sections to IAA solutions of varied concentration. 48 hours incubation in dark. Initial length: 5.0 mm. Each point based on average of forty sections.

This was readily controlled by the use of penicillin G in a concentration of 16,000 units/100 ml.

In the experiments reported below, coleoptile sections were incubated in IAA solutions either in a floating or in a submerged condition. After 48 hours of incubation in a dark room at 26°C the growth of the sections was measured under a dissecting microscope. The results of a typical experiment are shown in figure 3. The submerged sections are more sensitive to the lower concentrations of IAA than are the floating sections, and reach a maximum growth at an IAA concentration of 1 mg/l. The floating sections, on the contrary, although they are less sensitive to lower IAA concentrations, do grow to approximately the same final length as do submerged sections in sufficiently high concentrations of IAA (20 to 30 mg/l). These results suggest the possibility that the superior growth of rice coleoptiles under water may depend on a decreased auxin destruction as compared to coleoptiles in air.

It may be noted that there is a considerable differ-

TABLE I

EFFECT OF THE AUXIN ANTAGONIST, 2,4-DICHLOROPHENOXY-ISOBUTYRIC ACID ON THE GROWTH OF RICE SEEDLINGS. SEEDS SUBMERGED IN EXPERIMENTAL SOLUTIONS TO A DEPTH OF 5 CM. INITIAL LENGTH OF COLEOPTILE IS 1–2 MM. 48 HOURS INCUBATION

| | CONCENTRATION (MG/L) OF ANTI-AUXIN | | | | | | | SEEDS PLACED ON MOIST FILTER PAPER |
|---|---|---|---|---|---|---|---|---|
| | 0.0 | 0.01 | 0.1 | 1 | 10 | 25 | 50 | |
| Length of coleoptile, mm ... | 25.3 | 20.9 | 21.3 | 20.7 | 3.2 | 2.0 | 1.7 | 10.3 |
| Length of mesocotyl, mm ... | 1–3 | 1–3 | 1–3 | 1–3 | 0 | 0 | 0 | 0 |
| Length of root, mm ..... | 3.2 | 3.2 | 3.4 | 1.4 | 0 | 0 | 0 | 27.6 |
| Number of roots ..... | 1 | 1 | 1 | 1 | .. | .. | .. | 2.8 |

ence in growth between submerged and floating sections placed in water alone. This is true even though the same initial plant material was used for the two conditions.

This growth as well as the growth of decapitated rice coleoptiles under water is, however, undoubtedly due to endogenous auxin since it is completely inhibited by competitive auxin antagonists. For these experiments the anti-auxin, 2,4-dichlorophenoxy-isobutyric acid (4) was used. Germinating seeds with coleoptiles 1 to 2 mm long were submerged 5 cm deep in 2,4-dichlorophenoxy-isobutyric acid solutions of various concentrations. The anti-auxin in a concentration of 10 mg/l retards growth under water completely (table I).

EFFECT OF SOME RESPIRATORY INHIBITORS ON GROWTH UNDER WATER: It is known that the IAA induced growth of Avena coleoptiles is dependent on aerobic respiration and is inhibited by such inhibitors as KCN (1). This is true also of the growth of rice coleoptiles. Growth even of submerged coleoptiles may be totally inhibited by low concentrations of KCN.

For these experiments coleoptile sections prepared as described above were incubated with IAA solution (10 mg/l) containing 0, $10^{-5}$, $10^{-4}$, or $10^{-3}$ M KCN. Each solution was adjusted to pH 7.3. Both floating and submerged sections were used. After 24 hours incubation the length of the sections was measured. It was found as shown in table II that KCN at $10^{-3}$ M causes 90% inhibition of growth both of submerged and floating sections. The concentration of KCN which causes 50% inhibition of growth is approximately $10^{-4}$ M and is the same for sections in air and under submerged conditions. This suggests that the aerobic respiration may participate in the growth under submerged conditions as well as in the growth carried out in air.

TABLE II

INHIBITION OF GROWTH OF RICE COLEOPTILE SECTIONS BY KCN. ALL EXPERIMENTAL SOLUTIONS CONTAIN IAA (10 MG/L). pH = 7.3. 24 HOURS INCUBATION

| CONDITIONS OF INCUBATION | EXPT. NO. | PERCENT INHIBITION AT KCN CONCENTRATION (M) OF | | |
|---|---|---|---|---|
| | | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ |
| Floating | 1 | 25.0 | 43.7 | 86.2 |
| | 2 | 30.4 | 61.0 | 89.3 |
| | 3 | 38.1 | 35.7 | 90.0 |
| | AV. | 34.5 | 46.8 | 88.5 |
| Submerged | 1 | 30.3 | 36.9 | 88.2 |
| | 2 | 35.4 | 53.7 | 90.7 |
| | 3 | 34.6 | 41.3 | 89.8 |
| | AV. | 33.4 | 44.0 | 89.6 |

2,4-Dinitrophenol (DNP) is known to be a most effective inhibitor of auxin-induced Avena section growth (2, 3) and to act by uncoupling the linkage of plant respiration to phosphorylation (4). DNP exerts its effect by blocking the aerobic synthesis of adenosine triphosphate (ATP) but is without effect on anaerobic, fermentative, ATP production. To test the effects of DNP on rice coleoptile growth, the inhibitor was added in various concentrations (0, 2, 5 and 10 mg/l) to IAA solutions (10 mg/l) adjusted to pH 5.0. The results (table III) show that 2 mg/l of DNP gives 80–90% inhibition of growth both with the submerged and with the floating materials. No difference in sensitivity to DNP was observed between submerged and floating sections. Again, the growth under water appears to depend on aerobic respiration and on the energy released by the respiratory oxidations. It appears quite unlikely that it can depend in any important manner on energy released by fermentation.

IAA DESTROYING ACTIVITY OF THE RICE COLEOPTILE: The fact that submerged coleoptiles are much more sensitive to applied auxin than are floating coleoptiles suggests the possibility that in the latter auxin destruction may play a prominent role. To test this possibility the effect of floating and submerged coleoptiles on the disappearance of exogenous auxin was first tested. Solutions of IAA were incubated with

TABLE III

EFFECT OF 2,4-DINITROPHENOL ON GROWTH OF RICE COLEOPTILE SECTIONS. ALL EXPERIMENTAL SOLUTIONS CONTAIN IAA (10 MG/L). pH = 5.0. 24 HOURS INCUBATION

| CONDITIONS OF INCUBATION | PERCENT INHIBITION AT DNP CONCENTRATION (MG/L) OF | | |
|---|---|---|---|
| | 2 MG/L | 5 MG/L | 10 MG/L |
| Floating ..... | 91.6 | 100 | 100 |
| Submerged ... | 88.2 | 100 | 100 |

TABLE IV

RESIDUAL IAA REMAINING IN SOLUTIONS AFTER INCUBATION WITH RICE COLEOPTILE SECTIONS. INITIAL CONCENTRATION OF IAA SOLUTION IS 20 MG/L

| CONDITIONS OF INCUBATION | RESIDUAL AMOUNT OF IAA AS PERCENT OF INITIAL | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | EXPT. 1 | | | EXPT. 2 | | EXPT. 3 | | |
| | No.* | 2 HRS | 5 HRS | No.* | 24 HRS | No.* | 24 HRS | 48 HRS |
| Sections submerged ..... | 96 | 100 | 100 | 2 | 47.8 | 2 | 65.9 | 32.8 |
| Sections floating ..... | 96 | 80.6 | 58.0 | 2 | 18.1 | 2 | 49.6 | 2.1 |

* Number sections per ml.

coleoptile sections under floating or submerged conditions and the residual IAA was determined by the colorimetric method of Tang and Bonner (16), using Salkowski's reagent. In each case 40 sections were incubated with 20 ml of IAA (20 mg/l) solution. As shown in table IV the floating sections destroy more IAA than the submerged sections. In a short period experiment, in which 2400 coleoptiles were incubated with 25 ml of IAA solution (20 mg/l) for 2 and for 5 hours, similar results were obtained.

Rice coleoptiles are also able to destroy rapidly IAA which has been infiltrated into the tissue and this destruction depends on the aeration of the sections. Thirty gm (fresh weight) of coleoptiles were prepared, and IAA solution (500 mg/l) was infiltrated into them. The material was rinsed with water and divided into four portions. Two lots were left between wet filter papers for 5 and 10 hours respectively. One lot was used for the determination of IAA at initial time, while the last lot was incubated under nitrogen for 24 hours. The samples were lyophilized and residual IAA in the tissue determined colorimetrically on the ether extract obtained in each case from 150 mg of lyophilized tissue. The data of table V show that the IAA initially present in the tissue (120 μg/100 mg dry weight) was almost completely destroyed after 10 hours incubation of the coleoptiles in air. The amount of IAA destroyed was however greatly decreased in the coleoptiles which had been incubated under conditions of reduced oxygen tension.

## DISCUSSION

The results described above suggest that the elongation of rice coleoptile under water is not a particularly peculiar type of growth, but that the mechanisms involved are not qualitatively different from those already known for the Avena coleoptile. Both auxin and aerobic respiration are required for growth of the rice coleoptile under water. An aberrant feature of the rice coleoptile does however appear to reside in its ability to destroy IAA at the oxygen tension which obtains in air while, at the lower oxygen tensions which obtain when the coleoptiles are immersed in water, auxin destruction is reduced, and the growth responses to both endogenous and to added auxin are increased. At the same time, however, aerobic respiration is limited under water by the amount of oxygen available to the tissue as has been shown by Taylor (17). The growth of the rice coleoptile under water is then determined by a compromise between at least two opposing factors, namely the lowered auxin destruction and the lowered aerobic respiration (fig. 4).

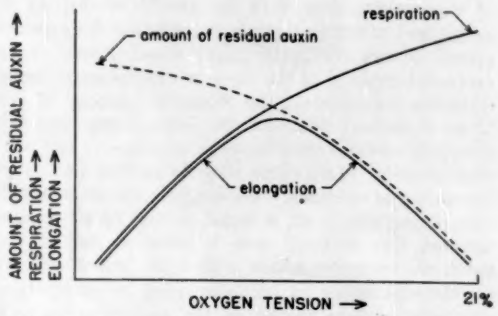


FIG. 4. Proposed scheme showing the hypothetical relationships between respiration, auxin destruction and elongation of the rice coleoptile. Auxin destruction is expressed in terms of residual (undestroyed) auxin which would remain in the tissue after a measured time and conditions which are uniform except in oxygen tension.

It has been suggested (17) that the energy released anaerobically by fermentation may be more efficiently used by the rice plant in its growth than is the energy liberated in oxidative respiration. This hypothesis was based on the fact that over the range of low oxygen concentrations in which rice exhibits greater germination and growth than wheat, rice also carries on a more active fermentation than does wheat. The results presented in this paper indicate, however, that

TABLE V

DESTRUCTION OF IAA AFTER INFILTRATION OF THIS MATERIAL INTO RICE COLEOPTILE TISSUE. RESIDUAL IAA IS GIVEN AS PERCENT OF INITIAL AMOUNT (120 μG/100 MG TISSUE DRY WEIGHT)

| | PERIOD OF INCUBATION | | | |
|---|---|---|---|---|
| | 0 | 5 HRS | 10 HRS | 24 HRS |
| In air ..... | 100 | 33.1 | 5.9 | ... |
| In nitrogen ... | ... | ... | .. | 50.1 |

fermentation cannot take the place of aerobic respiration in promoting growth of the rice coleoptile and that on the contrary growth is entirely dependent on cyanide sensitive oxidative energy coupling systems mediated in all probability through ATP.

Not only the coleoptile, but the stem and leaf sheath of the rice plant as well are known to elongate more rapidly under water than in air. This is frequently observed in the field as when older rice plants are submerged by water during floods. In addition there are grown in India and Burma varieties known as "deep water" or "floating" rice. These varieties are capable of growing in such a fashion as to keep pace with rises of water level which occur during the monsoon flood season. The shoots of these varieties may grow to heights of as much as 20 feet in response to the increase in water level (13). It will evidently be of interest to determine how far mechanisms similar to that described here for the coleoptile are involved in these further growth responses of the rice plant to water level.

## Summary

The work reported here concerns the well known fact that the rice coleoptile elongates more rapidly and more extensively under water than in air. Decapitation (removal of the apical tip) experiments carried out with plants in air or under water as well as experiments done with the growth of floating or submerged coleoptile sections suggest that the superior growth of rice coleoptiles under water depends on a decreased capacity of the tissue to destroy auxin under water as compared to the extensive capacity of the tissue to destroy auxin in air. Thus, submerged rice coleoptile sections grow more in response to low concentrations of added auxin than do sections floated on the surface of solutions. Although the maximal growth rate of sections in air is equal to that of submerged sections, this maximal rate is achieved only at an auxin concentration which is 20 to 30 times as great for sections in air as for submerged sections. Rice coleoptile sections floated on the surface of an IAA solution remove IAA from the solutions much more rapidly than do similar but submerged coleoptile sections. Similarly, IAA disappears much more rapidly from coleoptile tissue infiltrated with IAA and incubated in air than from similar coleoptiles incubated under reduced oxygen tension. The growth of the rice coleoptile depends on aerobic respiration even under conditions of complete submersion in water. The inhibition of growth by KCN as well as by DNP was found to be the same for floating and for submerged sections. It is suggested as a working hypothesis that growth of the rice coleoptile is determined by the interaction of two important opposing factors: (1) decrease of auxin destruction at lower oxygen tension, and (2) decreased aerobic respiration at low oxygen tension.

The writer wishes to express his indebtedness to Dr. James Bonner, to Dr. A. W. Galston and Dr. J. L. Liverman for their many helpful suggestions and criticisms during the course of this investigation.

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## BRIEF PAPERS

# AN IMPROVED INFILTRATION SERIES FOR STUDYING STOMATAL OPENING AS ILLUSTRATED WITH COFFEE[1]

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Several liquids or series of liquids have been utilized for the purpose of estimating the degree of stomatal opening by the so-called "infiltration" or "injection" method. Stahl (9) used a series consisting of paraffin oil, petroleum, and petroleum ether; Molisch (5) employed absolute alcohol, benzene, and xylol; Dietrich (2) used paraffin oil, castor oil plus turpentine, petroleum, and xylol. Since these series included only three or four liquids of different surface tensions, they gave rather rough estimations of stomatal aperture. Schorn (8) attempted to increase the accuracy of the infiltration method by using a larger series of liquids, which was prepared with isobutyl alcohol and ethylene glycol in eleven mixtures varying from pure isobutyl alcohol to pure ethylene glycol in steps of 10% by volume. As pointed out by Oppenheimer and Mendel (7), pure isobutyl alcohol does not penetrate stomata of small aperture and for that reason this series cannot be utilized for determining slight changes in the opening of such stomata.

The authors have obtained very satisfactory results in estimating the degree of stomatal opening of several plant species by using infiltration series prepared with Nujol mineral oil diluted with xylol, kerosene, *n*-dodecane, *n*-tetradecane, or Bayol-D.[2] All of these materials, except Nujol, infiltrate the leaves when the stomata are only slightly open. Nujol does not penetrate even wide open stomata, except for plants with particularly large stomata.

Series of ten or eleven concentrations were used in steps of 10% by volume from pure xylol (or *n*-dodecane, *n*-tetradecane, etc.) to pure Nujol. Steps of less than 10% concentration can be used for greater precision. This has proven especially suitable for plants with relatively small stomata, such as cacao, which is not penetrated by solutions containing more than 80% Nujol.

All of the materials which have previously been recommended for the infiltration technique, including xylol and kerosene, are toxic and cause necrotic spots where the drops are applied. This becomes a distinct disadvantage when it is desirable to utilize the same leaves for periodic tests of stomatal aperture, or when the number of leaves is limited, as in nutrient culture work with small plants. The pure hydrocarbons, *n*-dodecane and *n*-tetradecane, and the highly refined white oil, Bayol-D, are not toxic to most plants (3). The authors found that the pure hydrocarbons were toxic to bean leaves, although Bayol-D caused no injury. Neither the oil nor the hydrocarbons injured cacao, coffee, cassava, citrus and rubber. Nujol did not produce necrotic injury, but it evaporated very slowly from the leaf. It caused no injury to cacao and coffee, but concentrations above 80% caused some injury to beans.

Figures 1 and 2 illustrate results obtained with the infiltration series proposed by the authors. These data represent the cycle of stomatal opening of coffee leaves both in sunlight and in shade during two consecutive days (Oct. 15 and 16, 1952). These data were obtained with the series prepared with *n*-dodecane and Nujol in ten concentrations ranging, in order of decreasing penetrability, from 100%, 90%, 80%, etc., to 10% *n*-dodecane. Each point of the curve represents an average of at least five determinations on different leaves. The plants were about 4 years old and were growing in full sunlight. Only mature leaves, on the third to the sixth node from the branch tip, at about mid-height on the trees were used. The shaded leaves were those located on the shaded side of the plants. Light intensity was measured by means of a Weston sunlight meter, model 614.

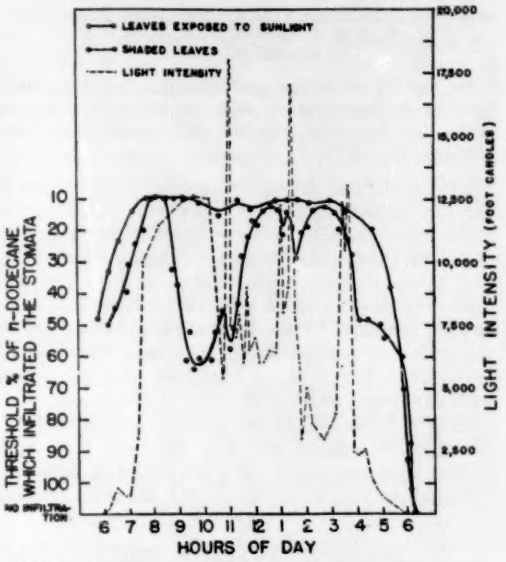


FIG. 1. Cycle of stomatal opening of coffee leaves, both in sunlight and in shade, as determined by the infiltration technique with the series *n*-dodecane-Nujol (October 15, 1952).

The results show that coffee leaves exposed to direct sunlight decreased their stomatal aperture dur-

[1] Received January 19, 1953.

[2] The three latter materials were provided through the courtesy of the Standard Oil Development Company, Linden, New Jersey.

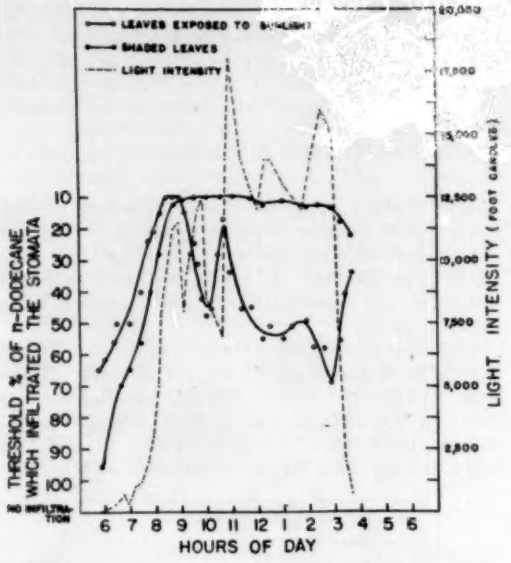


FIG. 2. Cycle of stomatal opening of coffee leaves, both in sunlight and in shade, as determined by the infiltration technique with the series *n*-dodecane-Nujol (October 16, 1952).

ing the periods of strong illumination in the middle of the day (more than about 8,000 fc). This partial closure was probably a result of excessive transpiration over water absorption (6). During the first day (fig. 1) the stomata began to close at 8:30 A.M. but opened again from 11:00 to 12:00 following a decrease in light intensity. Stomatal opening decreased during the periods of stronger illumination from 1:00 to 1:30, and 3:20 to 3:40 P.M., and attained complete closure with darkness around 6:00 P.M. Light intensity remained high the second day (fig. 2), until 3:00 P.M., and stomatal opening was kept low from 9:30 A.M. to that time, except for the short period of decreased illumination from 10:10 to 10:40 A.M., when opening increased. From 3:00 to 3:30 P.M., light intensity decreased and stomatal opening started increasing. The tests were discontinued at 3:30 because of rain. The stomata of leaves under shade remained wide open throughout both days. These results are in agreement with those obtained by Nutman (6), and Franco (3), by means of porometers.

A partial stomatal opening was noted at the beginning of both of the above tests, before the light intensity was measurable. This opening was probably caused by dew, as has been reported by Alvim (1) for other plants.

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# STUDIES ON THE NITROGENOUS NUTRITION OF RICE[1]

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The experiments reported here were carried out in order to clarify the old and controversial question of the relative merits of nitrate and ammonium salts in the nutrition of rice. The older literature is reviewed by Pardo (4) to explain the apparent superiority of ammonium salts. Bonner (1) has suggested that rice, at least in the seedling stages, is unable to reduce nitrates.

[1] Received January 23, 1953.

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Rice plants (*Oryza sativa* var. *Caloro*) were grown in the greenhouse by the nutrient culture method. For the preparation of the culture solution four stock N solutions were prepared as follows:

1. Nitrate-N only; (molar strength) + 0.01 ppm of molybdenum
2. $NH_4$-N only; (molar strength) + 0.01 ppm of molybdenum
3. Nitrate-N + $NH_4$-N (molar strength of each) + 0.01 ppm of molybdenum
4. Nitrate-N only; (molar strength) + 0.05 ppm of molybdenum

The other salts were uniform for the four solutions and were given as indicated in Hoagland's solution. Forced aeration was provided to a group of plants, and omitted in another. The pH was kept virtually constant at 6.0 throughout the experiment. Tomato plants of the Marglobe variety were grown in a parallel experiment for comparative purposes.

Two series of measurements and of chemical analyses were done at different stages of growth: nitrogen fractions, organic acids, sugars, mineral components, and the pH of the cell sap were determined by standard methods. However, we shall not give here the results of chemical analyses; they will only be discussed below. The fresh weight measurements from the second harvest appear in the table I. Statistical

TABLE I

FRESH WEIGHT OF 56 RICE PLANTS, IN GRAMS

| TREATMENTS | ROOTS | SHOOTS | TOTAL |
|---|---|---|---|
| 1. $NO_3$ + 0.01 ppm Mo + air .... | 228.0 | 416.0 | 664.0 |
| 2. $NH_4$ + 0.01 ppm Mo + air .... | 168.0 | 350.5 | 518.5 |
| 3. $\frac{1}{2}NO_3 + \frac{1}{2}NH_4$ + 0.01 ppm Mo + air ..... | 234.0 | 444.0 | 678.0 |
| 4. $NO_3$ + 0.05 ppm Mo + air .... | 220.0 | 448.0 | 668.0 |
| 5. $NO_3$ + 0.01 ppm Mo, no air .. | 225.6 | 456.0 | 681.6 |
| 6. $NH_4$ + 0.01 ppm Mo, no air .. | 221.6 | 432.0 | 653.6 |
| 7. $\frac{1}{2}NO_3 + \frac{1}{2}NH_4$ + 0.01 ppm Mo, no air ..... | 272.0 | 480.0 | 752.0 |
| 8. $NO_3$ + 0.05 ppm Mo, no air .. | 307.3 | 512.2 | 819.5 |

analyses revealed no significant difference among treatments 1, 3, and 4; on the other hand, treatment 8 is superior to all the other treatments at the 0.1% level.

Chemical analyses showed the following points of interest. First, in the first series of determinations which were carried out when the plants were 4 weeks old, a considerable accumulation of $NH_3$ was found in the $NH_3$-treated plants; this abnormal $NH_3$ concentration was not accompanied by a simultaneous rise on asparagine or glutamine content; this indicates that probably the usual $NH_3$ detoxication mechanism through amide formation is not active at that stage; the same plants in the second series of analyses, taken 1 month later, were verified to have a still higher $NH_3$ content and also a significantly higher amide fraction; we believe that this toxic excess of free and combined $NH_3$ is one of the factors responsible for the reduction in growth now verified. Second, $NO_3$-N was found to be present in the $NH_3$-treated plants; similar results have been recorded by Clark (2) and by Delwiche (3); but the former admitted the possibility of nitrification in his sand cultures, whereas Delwiche worked with material under catabolic conditions; in our case, however, no nitrification was detected in the solutions; since $NO_3$ was consistently found in both series of analyses, we are forced to admit that a reoxidation of reduced forms of N took place; we do not know the biological significance of this fact, but, as a working hypothesis we might assume that it is an auxiliary detoxication mechanism for $NH_4$ accumulation.

From the present experiments the following conclusions can be drawn. First, the reduction in growth suffered by the $NH_3$ plants was due not only to the toxic accumulation of large amounts of $NH_4$ but also to implications in the photosynthetic and respiratory mechanisms as revealed by the sugars and organic acids determinations. Second, the different growth response obtained with the withdrawal of forced aeration throws some light on a few disagreements in the literature on rice nutrition. Third, the influence of the higher amount of Mo supplied cannot be merely confined to nitrate reduction—our present knowledge of the roles of Mo on nitrogen, and possibly carbohydrate metabolism is still very inadequate indeed. Fourth, the harmful influence of aeration gives rise to a question about the possible significance of this fact on ion accumulation; this problem is now being studied with the aid of the radioisotopic technique.

The author is indebted to Prof. P. R. Stout, Chairman of the Department, for his advice and interest during the course of this investigation.

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# THE INHIBITING EFFECT OF ROOT TIPS ON THE ELONGATION OF EXCISED PISUM EPICOTYLS CULTURED IN VITRO[1]

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Inhibition of root growth by substances present in roots has been reported by Goodwin and co-workers (1, 2), who found that growth of Avena roots is inhibited by scopoletin, a coumarin derivative which they find to be normally present in non-elongating portions of these roots.

The present paper is a report of inhibition of stem elongation by a substance or substances present in roots of *Pisum sativum*.

Went (5) has presented evidence that a stem growth hormone, for which he proposed the name "caulocaline," originates in the roots or lower portions of the plant and is translocated upward. Skoog (3) has cultured excised pea epicotyls in vitro and has demonstrated that they elongate readily but not indefinitely on standard White's medium containing the organic supplements used by Skoog and Tsui (4). Indoleacetic acid (IAA) added to the nutrient medium was shown to inhibit rather than promote growth of the epicotyls in all but exceedingly low concentrations (3). Cultures of this type were therefore chosen as test objects for a further study of stem growth factors in roots (Howell and Skoog, in press). It was expected that the elongation of the pea epicotyls would be stimulated or the period of their elongation extended if excised root tips were placed on the surface of the nutrient medium. Contrary to expectations, the root tips exert a strong inhibitory effect as shown by results of experiments presented below.

## MATERIALS AND METHODS

Seeds of *Pisum sativum*, var. Alaska, were sterilized in 0.1% $HgCl_2$ solution, then rinsed four times with sterile distilled water, and soaked overnight in sterile distilled water. The peas were then removed, immersed in 95% alcohol and finally rinsed with three changes of sterile distilled water, after which the epicotyls were excised aseptically and planted on the surface of sterile culture media. The medium used was that of Skoog and Tsui (4), adjusted to pH 4.0, containing 2% sucrose and solidified with 0.8% agar. The medium was pipetted into glass screw cap vials. In the earlier experiments 10 ml and in the later ones 5 ml of medium were used per vial.

Root tips were obtained from peas which had been surface-sterilized as above and germinated in the dark in Pyrex baking dishes which were heat-sterilized prior to use. After three days, when the roots of most of the peas were 3 cm long or longer, they were excised, trimmed to 1 cm lengths, and planted on the surface of the agar in the vials. This operation was carried out aseptically.

Ether and aqueous extracts were obtained by allowing roots excised from peas germinated as above to stand in ether or water at 4° C for three days. The ether extracts were evaporated to dryness and the residues taken up in equal volumes of water.

The vials containing the epicotyls and roots were placed on a shelf over the laboratory bench or on shelves in hallway cabinets during the culture period.

## RESULTS AND DISCUSSION

In a preliminary experiment, three epicotyls and six excised root tips were planted in each vial. The growth of the epicotyls was reduced to only 18% of that of the controls without roots present. The experiment was repeated with various numbers of epicotyls and root tips per vial. The results (table I) show that as the relative number of root tips per vial was increased, the inhibition of the epicotyls was also increased. The systematic nature of this increase is seen more readily from the curve of figure 1, in which the ratio: number of root tips/number of epicotyls is plotted against the per cent inhibition. The inhibition does not seem to be due to crowding or to competition for nutrients since there is no inhibition when the number of epicotyls per vial is increased while the number of roots is held constant.

The inhibitory activities of ether and aqueous extracts (table II) show that the inhibitor is ether soluble. The water extract also gave a considerable inhibition, but the inhibiting substances were largely removed by ether extraction of the aqueous extract.

It is to be noted that the inhibitor evidently acts at least in part by inhibiting the development of roots on the epicotyls. In the vials containing excised root tips, rooting of the epicotyls was drastically reduced,

TABLE I

INHIBITORY EFFECT OF EXCISED ROOT TIPS OF PEA SEEDLINGS ON ELONGATION OF EXCISED PEA EPICOTYLS

| No. of vials | Number of explants in each vial | | Growth of epicotyls after 16 days | | |
|---|---|---|---|---|---|
| | Epicotyls | Root tips | Mean increase (mm) | Increase % | No. rooted % |
| 3 | 1 | 0 | 7.3 | ... | .. |
| 3 | 3 | 0 | 6.6 | ... | .. |
| 4 | 6 | 0 | 7.7 | ... | .. |
| 10 | Avg. 1–6 | 0 | 7.3 | 100 | 43 |
| 10 | 6 | 1 | 6.4 | 80 | 8 |
| 10 | 3 | 1 | 5.9 | 70 | 0 |
| 10 | 1 | 1 | 5.5 | 61 | 0 |
| 10 | 1 | 3 | 5.4 | 59 | 0 |
| 10 | 1 | 6 | 4.8 | 46 | 20 |

[1] Received January 28, 1953.

[2] Present address: U. S. Regional Soybean Laboratory, Urbana, Illinois.

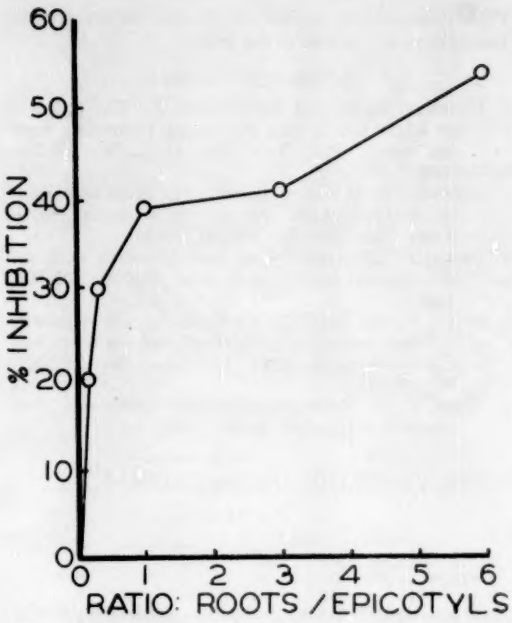


FIG. 1. Inhibition of elongation of excised pea epicotyls by excised pea root tips as a function of the ratio of number of root tips to number of epicotyls.

and in the cultures with the ether extractable material from roots incorporated in the nutrient medium, rooting was completely suppressed. In both these groups, epicotyl elongation was also markedly inhibited. Since the water extracts from which the ether soluble fraction had been removed caused little or no inhibition of either rooting or epicotyl elongation, it appears that the observed inhibition of epicotyl elongation is at least in part a secondary effect resulting from the inhibition of root formation.

Inhibition similar to that obtained from the pea root tips was also obtained from root tips of greenhouse-grown *Vicia faba* plants, indicating that the inhibitor is not limited to pea roots but may be of general occurrence.

The distribution of the inhibitor within the root was determined by testing extracts of three successive one-centimeter segments. The results, plotted in figure 2, show that the concentration of the inhibiting substances decreases rapidly with the distance from the tip of the root and that the third cm from the tip yields relatively very little inhibitor.

Since the inhibitor is ether soluble, the ether extracts were tested for auxin activity by the standard Avena curvature test after being concentrated to 1/32 the volume in which they effectively inhibited epicotyl growth when added to the nutrient medium. Since the Avena tests were completely negative, the auxin content of the ether extracts must have been $< 10$ $\mu$g/l. IAA equivalents in the test solution or $< 10/32$ $\mu$g/l. IAA equivalents in the nutrient medium. The concentration of IAA required in the nutrient medium to obtain significant inhibition of epicotyl growth is of the order of 10 $\mu$g/l. It is clear, therefore, that the inhibitor of epicotyl growth is not auxin. This is also indicated by the fact that IAA concentrations which inhibit epicotyl growth tend to promote rather than inhibit their rooting. The inhibitor, therefore, is apparently not an auxin which is active in this test.

TABLE II

INHIBITORY EFFECT OF EXCISED ROOT TIPS AND OF EXTRACTS OF ROOT TIPS ON ELONGATION OF EXCISED PEA EPICOTYLS

| TREATMENT | GROWTH AS % OF CONTROL | | PER CENT ROOTED | |
|---|---|---|---|---|
| | 24 D | 54 D | 24 D | 54 D |
| Control | 100 | 100[a] | 57 | 57 |
| 5 root tips/vial | 33 | 38 | 17 | 24 |
| Ether extr of 5 root tips/vial | 35 | 23 | 0 | 0 |
| Water extr of 5 root tips/vial | 60 | 70 | 54 | 50 |
| Ether extr of water extr | 31 | 24 | 0 | 0 |
| Water extr after ether extraction | 71 | 86 | 35 | 44 |

[a] Initial length of epicotyls 2.7 mm. Final length of controls 13.7 mm. Ten vials per treatment; 2 epicotyls per vial.

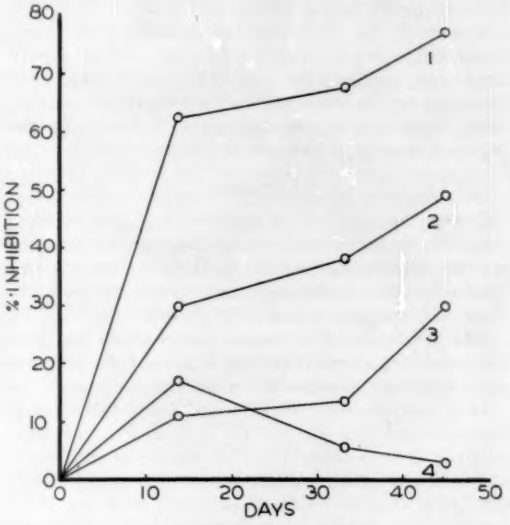


FIG. 2. Inhibiting effect of excised root tips and of extracts of various segments of root tips on elongation of excised pea epicotyls. Curve 1, 5 *Vicia faba* root tips per vial; 2, ether extract of terminal centimeter of 5 pea root tips per vial; 3, ether extract of second centimeter of 5 pea roots per vial; 4, ether extract of third centimeter of 5 pea roots per vial.

## SUMMARY

The elongation of excised epicotyls of Alaska peas in sterile culture is inhibited by root tips placed on the surface of the agar medium or by extracts from the tips added to the nutrient medium.

The degree of inhibition increases with the ratio: number of root tips/number of epicotyls.

The inhibitor is present in highest concentration in the terminal centimeter of the roots and decreases in amount with distance from the tip.

The inhibitor from roots is ether soluble, but is inactive in the Avena test. It differs from auxin also in that it inhibits root formation. The latter effect may be at least partially responsible for the inhibition of epicotyl growth.

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Tests of extract for auxin activity were kindly conducted by Mr. John B. Baker of the University of Wisconsin.

The author wishes to express his appreciation to Prof. Folke Skoog for his advice and encouragement throughout the course of the work.

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# LEAF AND AIR TEMPERATURES UNDER FREEZING CONDITIONS[1,2]

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Air temperature may give a poor indication of the freezing injury which leaves will suffer. Type and condition of the plant and the meteorological conditions affect the extent of the damage. Under certain conditions, particularly calm clear nights, leaf temperatures can be considerably lower than air temperatures, especially air temperatures recorded by the Weather Bureau in a shelter at 135 cm.

## Materials

During the night of October 5–6 leaf and air temperatures were recorded, using 30-gauge copper constantan thermocouples with a Brown electronic recording potentiometer which recorded 16 different thermocouples in a period of 80 seconds.

The plants used were tomato plants which had been subjected to cold temperatures, but until the previous night had been protected from freezing.

Leaf temperatures were recorded by threading a thermocouple into the leaf (3, 4, 7, 8, 9). Air temperature was recorded 1 cm above one of the leaves and in a louvred instrument shelter at a height of 135 cm.

## Results

The recorder was run continuously the night of October 5–6. In figure 1, data taken from the chart at 15-minute intervals are plotted. Each point is the average of 3 thermocouples. Variability between thermocouples was very small. They show the leaves to have been 3 to 4.5°C cooler than air temperature in the shelter when the sky was clear. Shortly before 9 P.M. a cloud cover moved in, and the difference became less than 3°C. Early in the morning when the cloud cover was about 0.5 the difference was steady at slightly above 2°. When compared with air temperature 1 cm above the leaf, the leaf was found to be about 1°C cooler. Waggoner and Shaw (8) have stated that the absolute difference between a leaf and a thermocouple 1 cm above it is biased downward during periods of outgoing radiation. The 1°C difference measured is probably a slight underestimate of the true difference. Leaf number 2, which had suffered some freezing injury the previous night, maintained a slightly warmer temperature than the normal leaves, except during the period of leaf warming at 8:15 P.M.

A sudden rise in leaf temperature, considered to indicate the time of freezing, was found between 8 and 8:30 P.M. The leaf injured the previous night showed a gradual rise in temperature. Leaf 1 showed a rise of almost 2°C after having cooled to between −4.0 and −4.5°C. Leaf 3 did not show the rapid rise in temperature. During the night leaf temperatures dropped to −8° and the next morning all leaves were dead. In the upper right corner of figure 1, the temperature curve for leaf #1 is plotted on a time interval of 160 seconds.

## Discussion

Brown and Escombe (1) speak of heat losses by both conduction and radiation, but Curtis (7) pointed out that they and others (10) have failed to realize that leaves may become cooler than the air about them due to radiation to cooler objects or to space. Waggoner and Shaw (8) have shown that energy losses at night due to transpiration may be very small. Since the leaf is cooler than the air, conduction must result in a net movement of heat toward the leaf and the main loss at night must be due to radiation.

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[2] Journal Paper No. J-2237 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 996.

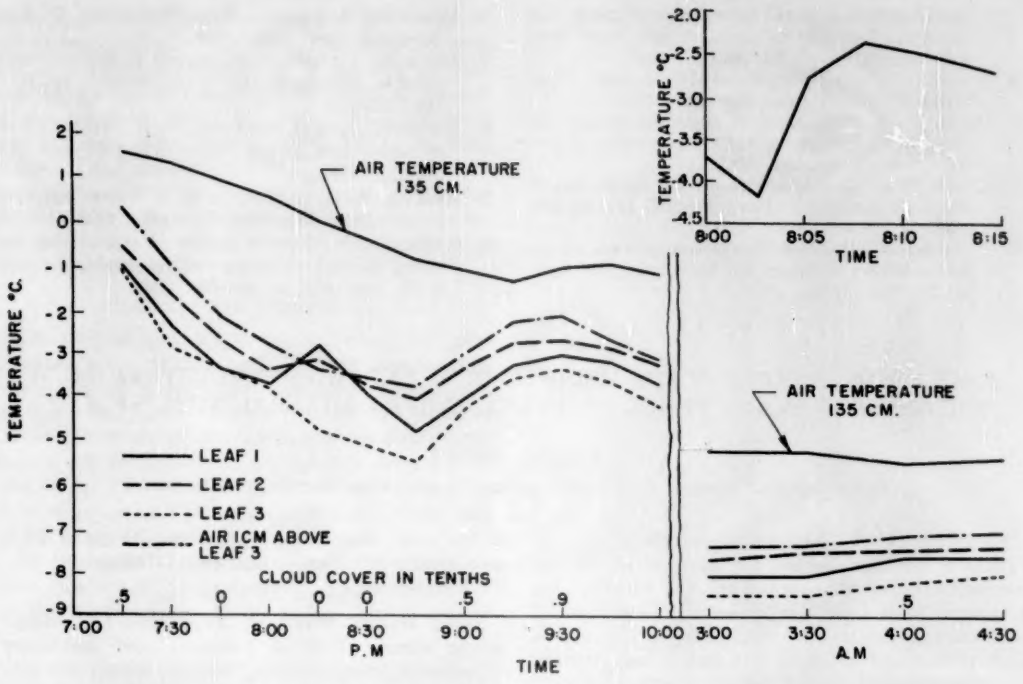


FIG. 1. Leaf and air temperatures the night of October 5–6.

Brunt (2) has suggested the following empirical formula for the net upward radiation from the leaf to a clear sky:

$$U = \sigma A^4(0.56\text{–}0.8\sqrt{e})$$

where $\sigma$ is Stefan's constant, $A$ absolute temperature of the leaf and $e$ vapor pressure in millibars at a height of 150 cm. With increasing sky cover Lauscher (5) has shown that the values decrease to 33% of clear sky radiation when cloud cover goes from 0 to 0.9. Using the 7 P.M. reading of 0°C and a vapor pressure of 2.7 millibars, $U$ is 0.194 cal/min/cm² for a clear sky. Small changes in temperature have little effect on the magnitude of $U$. The difference in leaf and air temperature was caused by the favorable conditions for strong outgoing radiation. The 3 to 4.5°C difference was reduced to about 2°C when the sky became partly cloudy. When air temperatures were measured 1 cm from a leaf the difference between leaf and air temperature was slightly over 1°C. This difference is smaller because the air surrounding the leaf is rapidly cooled by the cold leaf. At a height of 135 cm much larger differences were found; and freezing of leaves can occur when these air temperatures are near or slightly above freezing. There was a 30-minute lag between the cooling of the air at 135 cm and the cooling of the leaves. At 8:45 P.M. the leaf temperature showed a rise in temperature, but the air continued to cool until 9:15 P.M.

It frequently happens that water or plant sap which is gradually cooled begins to freeze below the freezing point. With the initiation of ice crystals the heat of fusion is released, resulting in a rise in temperature. Meyer and Anderson (6) state the actual freezing points of plant tissues are seldom below –5°C. Of the leaves measured, one leaf, #3 did not show a sudden rise in temperature at any time. Evidently there was no undercooling in this leaf. Leaf 2 which had been injured the previous night showed a gradual rise in temperature. Leaf 1, and another leaf for which the curve is not shown cooled to –4.5°C then showed a sudden rise of 1.5°C. These tomato leaves were evidently undercooled to –4 to –4.5°C before ice crystals were formed.

## SUMMARY

Air temperatures in a standard instrument shelter at 135 cm were found to be as much as 3 to 4.5°C warmer than leaf temperatures of tomato plants when the conditions were favorable for strong upward radiation from the leaf. With almost a complete overcast this difference was reduced to 2°C. A leaf was about 1°C colder than the air 1 cm above the leaf. Certain leaves measured cooled to between –4 to –4.5°C then showed a sudden rise of almost 2°. This was believed to be due to the heat of fusion liberated when freezing occurred. Other leaves did not show this undercooling.

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# SOME OBSERVATIONS ON THE REDUCTION OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE IN PLANT TISSUE AS INFLUENCED BY MINERAL NUTRITION [1, 2]

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Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to the insoluble red formazan, which was developed by Lakon (9) as a test for seed viability; has proven of some indicative value, in tissue tests, of nutritional irregularities. Reduction of the dye is most pronounced at nodes (5) and in meristematic tissues irrespective of their position in plants. Hewitt and Agarivala (7) have observed tissues of plants grown with low molybdenum to show much less reduction of TTC than normal plant material. They report the distribution of molybdenum in plants grown with an adequate molybdenum supply as similar to the distribution of the precipitated formazan.

In previous experiments in which enzymatic activity was studied as indicative to copper or iron deficiencies and their interactions in plants (3, 4), fifteen plant species were grown on five soil treatments of varying iron- and copper-supplying capacity. Seven plant species developed lime-induced chlorosis but did not develop copper-deficiency symptoms; four plant species developed severe copper-deficiency symptoms but did not develop lime-induced chlorosis; two plant species showed partial symptoms of both types of chlorosis and two plant species grew well on all five soil treatments.

A marked difference in the ascorbic acid oxidase activity of these plants was observed between treatments. The ascorbic acid oxidase activity was much greater in the nodes than in the leaves of both corn and wheat and was found to be indicative of the external copper supply. Iron accumulated in the nodes of copper-deficient corn but did not accumulate in the nodes of corn grown on the calcareous soil. The ascorbic acid oxidase activity in the latter nodes was fifty times greater than it was in copper-deficient corn nodes. This marked difference in activity localized in the nodal areas of corn prompted the use of TTC as a further index to the nutritional irregularity.

[1] Received April 13, 1953.

[2] This project was supported in part by the U. S. Atomic Energy Commission.

## Methods and Materials

Minn. Hybrid #800 corn (*Zea Maize*), Thatcher spring wheat (*Triticum sativum*), and cockleburs (*Xanthium pensylvanicum*) were grown on five different soil treatments with various levels of iron and copper supplying capacity. These soil treatments were: (1) Calcareous soil, pH 7.8; (2) 2/3 calcareous 1/3 organic soil (by volume), pH 7.5; (3) 1/3 calcareous 2/3 organic, pH 7.2; (4) organic soil, pH 6; and organic soil plus added $CuSO_4 \cdot 5H_2O$. The calcareous soil was a Millville silty clay loam from Logan, Utah; and the organic soil was from the Michigan State College Experimental Muck Farm, East Lansing, Michigan. The soils were fertilized as described previously (3), and after thorough intermixing of the fertilizer with the soil, the respective treatments were placed in two-gallon glazed pots, equally moistened, and allowed to stand for one week before planting.

The lime-induced chlorotic and copper-deficient corn plants were harvested before the tassels became visible. The wheat plants were harvested just before the heads became visible and after severe copper-deficiency symptoms had developed in treatments 3 and 4. The cocklebur plants were harvested after the burs had developed.

The corn plants were cut off at the surface of the soil, and the cut ends immediately placed in a brown bottle, in subdued light, containing a 0.5% aqueous solution of TTC. The upward movement of the dye was observed by its reduction to the red formazan in the leaves, especially the veins, of the plant. Some plants were left in the solution for about six hours, and then the stalks were cut open to observe to what extent and where TTC had been reduced. Other plants were left in the solution for twenty-four hours before observations were made.

The entire wheat plant was harvested including as

much of the root systems as could be effectively removed from the soil. The roots of these plants were washed in water and then placed in the TTC solution and the upward movement and reduction of the dye determined as for corn. In addition, nodes and roots were cut from wheat plants harvested from the same treatment and placed in the TTC solution. The burs from the cocklebur plants were cut open and placed in this solution. Such experiments have been carried out three times for corn; twice for wheat, and once for the cockleburs.

## Results and Discussion

The fruit of corn, wheat and cockleburs was most affected by copper deficiency. In each case the ears, heads and burs formed, but they did not continue to develop and mature. Figure 1 shows the nature of the copper-deficiency symptoms with the undeveloped burs of the cocklebur plant. Figure 2 shows the extent of the TTC reduction in the nodes of corn. The least reduction of TTC occurred in plants which were grown on the copper-deficient organic soil.

In the stalks of corn and wheat which were cut open, the red formazan precipitate was observed to be located principally in the nodes. The internodes showed no, or very little, reduction of the dye. The red formazan precipitate was most pronounced in the veins of leaves.

Fig. 1. Comparative bur development on cockleburs grown on soils (left to right): copper-deficient organic soil and organic soil to which $CuSO_4 \cdot 5H_2O$ was added. Greatest reduction of 2,3,5-triphenyltetrazolium chloride occurred in the burs which were grown on the soil to which $CuSO_4 \cdot 5H_2O$ was added.

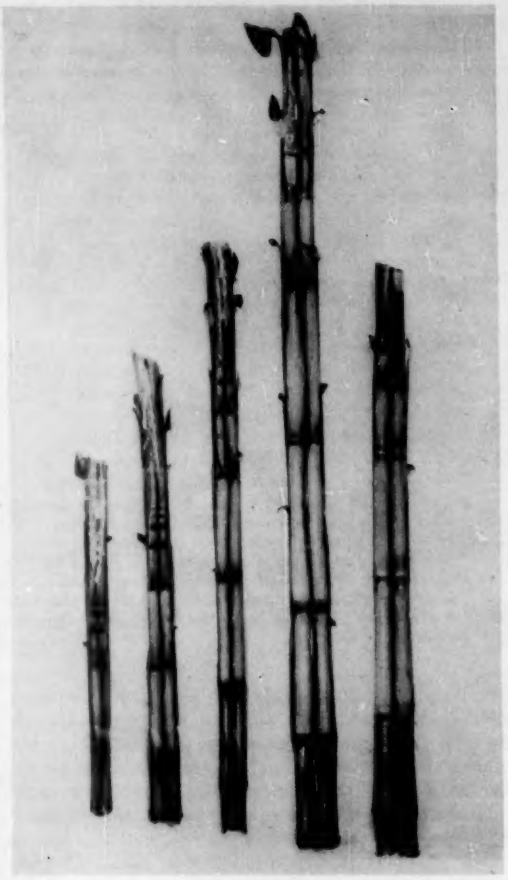

Fig. 2. Comparative growth of corn and reduction of 2,3,5-triphenyltetrazolium chloride (TTC) in the nodes of corn grown on the five soil treatments (left to right): (1) calcareous soil, (2) $\frac{2}{3}$ calcareous, $\frac{1}{3}$ organic soil (by volume), (3) $\frac{1}{3}$ calcareous, $\frac{2}{3}$ organic, (4) organic soil plus $CuSO_4 \cdot 5H_2O$, (5) organic soil. The greatest reduction of TTC occurred in plants grown on the calcareous soil; the least reduction occurred in the copper-deficient plants, soil 5. The latter plants showed some browning in the nodal areas, but very little of the red formazan occurred in these plants.

Table I includes data of the ascorbic acid oxidase and catalase activity taken from previous experiments (3, 4), iron and copper concentrations as determined spectrographically by Dr. A. W. Specht and J. W. Resnicky, and comparative ratings of TTC reduction in corn and wheat.

The comparative ascorbic acid oxidase activity, copper concentration, and TTC reduction ratings for corn and wheat were similar when grown on the five treatments. The catalase activity and iron concen-

TABLE I

COMPARATIVE ENZYMATIC ACTIVITIES OF ASCORBIC ACID OXIDASE AND CATALASE, COPPER AND IRON CONCENTRATIONS, AND THE REDUCTION OF TTC IN TISSUE OF MINN. HYBRID #800 CORN AND THATCHER SPRING WHEAT

| SOIL TREATMENTS [a] | COMPARATIVE AAO ACTIVITY [b] | | Cu * PPM LEAVES DRY WT | COMPARATIVE RATINGS OF TTC REDUCTION IN NODES † | COMPARATIVE CATALASE ACTIVITY [c] IN LEAVES | Fe * PPM LEAVES DRY WT |
|---|---|---|---|---|---|---|
| | LEAVES | NODES | | | | |
| | | | *Corn* | | | |
| 1 | 51 | 371 | 10 | + + + + + | 24 | 81 |
| 4 | 30 | 171 | 9 | + + + + | 70 | 74 |
| 2 | 19 | 261 | 11 | + + + + | 62 | 110 |
| 3 | 19 | 91 | 3 | + + + | 125 | 93 |
| 5 | −4 | 7 | 3 | + | 193 | 150 |
| | | | *Wheat* | | | |
| 1 | 23 | 163 | 11 | + + + + + | 427 | 85 |
| 4 | 24 | 118 | 25 | + + + + | 458 | 110 |
| 2 | 15 | 49 | .. | + + + | 393 | 110 |
| 3 | 7 | 32 | .. | + + + | 310 | 110 |
| 5 | 5 | 15 | 2 | + | 241 | 95 |

* Determined spectrographically by Specht and Resnicky.

† This is a visual comparison and rating of the TTC reduced in the nodes of corn and wheat. The larger the number; the greater the reduction of TTC.

[a] Soil treatments: (1) Calcareous soil, (2) ⅔ calcareous, ⅓ organic (by volume), (3) ⅓ calcareous, ⅔ organic, (4) Organic soil plus $CuSO_4 \cdot 5H_2O$, (5) Organic soil.

[b] μl of oxygen taken up/hr × 2 ml aliquot of plant sap extracted from 10 grams fresh weight sample using ascorbic acid as substrate. Sample pulverized and extracted with 5 ml of demineralized water.

[c] Volume of $O_2$ evolved (ml)/5 min × gram fresh weight of plant material.

tration differed between the two plants in some treatments. The catalase activity was comparatively twenty times greater in wheat than in corn when grown on the calcareous soil. Corn showed symptoms of lime-induced chlorosis; wheat did not show these symptoms. Yields were less and copper-deficiency symptoms were much greater for wheat than for corn when grown on the copper-deficient soils. The addition of copper to the organic soil increased the ascorbic acid oxidase activity in both corn and wheat, but it decreased the catalase activity in corn and increased it in wheat. The iron concentration decreased in the corn leaves but did not vary appreciably in the wheat leaves with the addition of copper to the organic soil.

The effect of copper on the fruit development in cockleburs was shown very clearly in the failure of the burs to develop (fig. 1) when copper was deficient. Plants grown on the calcareous soil, and organic soil to which copper was added, had a higher ascorbic acid oxidase activity and copper concentration than the plants grown on the copper-deficient soils (4). The green weight of burs developed on these plants was ten times greater than that harvested from the two most copper-deficient treatments. TTC was reduced to a much greater extent in the large developing burs of normal plants than in the undeveloped burs of copper-deficient plants.

Reduction of TTC appeared to be greatest in the meristematic regions of the plant. As the corn and wheat became more mature, the older nodes showed less reduction of TTC. Porter *et al.* (10) have indicated that the development of the non-diffusible red formazan in a specific tissue is presumably an indication of the presence of active respiratory processes in which electrons are transferred to TTC. That wheat germ contains cytochrome oxidase was shown by Brown and Goddard (2) who found that the oxidase was inhibited by HCN, $NaH_3$ and photoreversibly by CO. Allen and Goddard (1) observed that mature leaves of wheat are not inhibited by these same poisons. In differentiation of the leaves, Goddard (6) concludes there has been a change in metabolism that either blocks the sensitivity of the cytochrome oxidase to these poisons or a new oxidase system has been developed which replaces the cytochrome oxidase. James (8) more recently has found the cytochromes, cytochrome oxidase and ascorbic oxidase present in barley embryos; polyphenol oxidase was absent. The reaction mechanism in young roots was found to be quite different from that of the embryos. The cytochrome system and polyphenol oxidase were both absent in young roots, and the only oxidase yet identified was ascorbic oxidase. These data indicate that so fundamental an attribute of the living cell as its transfer of electrons to atmospheric oxygen may suffer changes of mechanism even at a time when the cell is in full vigour of growth.

In this study, the reduction of TTC is best correlated with ascorbic acid oxidase activity and copper concentration than with catalase activity or the concentration of iron in these plants.

## CONCLUSIONS

Copper deficiency retards the development of reproductive organs of corn, wheat and cockleburs. The

dye 2,3,5-triphenyltetrazolium chloride was only weakly reduced in the copper-deficient plants. Maximum reduction of TTC was obtained in plants grown on a naturally calcareous soil or an organic soil to which copper was added.

Comparative ascorbic acid oxidase activity, catalase activity and the concentration of copper and iron in corn and wheat showed that the reduction of 2,3,5-triphenyltetrazolium chloride in these plants could best be correlated with copper than with iron nutrition.

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## THE EXTRACTION AND COLORIMETRIC ESTIMATION OF INDOLE-3-ACETIC ACID AND ITS ESTERS IN DEVELOPING CORN KERNELS[1, 2, 3]


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The chemical identification of auxin, indole-3-acetic acid (IAA), isolated from the ethanol extracts of immature corn kernels by Haagen-Smit, Dandliker, Wittwer and Murneek (3) initiated considerable interest in both the quantitative and qualitative aspects of the auxin economy of corn kernels during their ontogeny. Avery, Berger, and Shalucha (1), Wittwer (8) and Stehsel (6) have studied these changes in auxin content during the development of the corn kernel. Recently Redemann, Wittwer and Sell (5) have reported the isolation of ethylindole-3-acetate from corn kernels and found that this substance was approximately 100 times more effective than IAA as a tomato fruit-setting agent.

To supplement biological methods of auxin assay, a colorimetric method was first described by Mitchell and Brunstetter (4). This procedure was later improved by Tang and Bonner (7), and by Gordon and Weber (2). Essentially, the method takes advantage of the red coloration formed through the mild oxidation of IAA.[4] Although sensitive, it is not always suitable for the determination of the auxin concentration in plant materials because of the presence of other pigments and the frequent formation of relatively stable colloidal suspensions. Since difficulties are encountered with extracts of plant materials, it seemed advisable to describe further refinements of the colorimetric methods developed in this laboratory and especially those procedures adapted for the quantitative determination of IAA and its esters in corn kernels during the various stages of development.


[1] Received April 27, 1953.

[2] Journal Article No. 1465 from the Michigan Agricultural Experiment Station, East Lansing, Michigan.

[3] This research was supported by the Horace H. Rackham Research Endowment of Michigan State College.


[4] This oxidation product will be described in another publication.

### Experimental

Colorimetric Measurements: The procedure is modified from that of Gordon and Weber (2), in that 0.05 M ferric chloride in 10% (by volume) perchloric acid was used to develop the red oxidation product, maximum intensity of which was attained in 45 minutes when held at 30°C. The 10% perchloric acid was used in preference to 35% to facilitate extraction in the final separation. The red color was quantitatively extracted with isobutyl alcohol, and the isobutyl alcohol layer centrifuged to remove the suspended water droplets. Since the red color is unstable in this medium, the time after extraction to reading was standardized at five minutes; the absorbancy was determined at 530 mμ on a Beckman D.U. spectrophotometer. Under these conditions, Beer's law was obeyed over a concentration range of 0 to 0.8 mg/25 ml isobutyl alcohol and an absorbancy index of 2.7 ml $(\text{cm mg IAA})^{-1}$.

Application of the Method to Plant Material: To ascertain the accuracy of the method, weighed amounts of IAA were added to 500 gm samples of immature corn kernels at the milk stage and macerated with ethyl acetate in a Waring blendor. The

resultant pulp was centrifuged, the solvent decanted, and the centrifugate washed 3 successive times with 25 ml portions of ethyl acetate. The combined ethyl acetate fractions were extracted with 25 ml of 2% $Na_2CO_3$. Two 10 ml aliquots of the aqueous layer were added to each of two 25 ml volumetric flasks. To one of the flasks 5 ml of 0.05 ferric chloride in 10% perchloric acid solution were added and both flasks made to volume with 10% perchloric acid. After permitting the reagent to react for 45 minutes, both solutions were extracted with 25 ml of isobutyl alcohol. The absorbancy of the color developed in the flask containing the iron solution was compared with the control having no iron solution. A similar experiment as a blank was made using corn kernels to which no IAA had been added. The readings obtained from the latter procedure were subtracted from those obtained by the addition of weighed quantities of IAA to the corn extracts. The results of these determinations are tabulated in table I. With this procedure esters of IAA do not interfere. However, if one refluxes the esters in 3% alcoholic KOH solution, the esters are completely hydrolyzed at the end of one hour and the same method may be used to determine the ester content in the corn kernels.

TABLE I

DETERMINATION OF VARIOUS QUANTITIES OF INDOLE-3-ACETIC ACID ADDED TO CORN KERNELS TO ESTABLISH THE VALIDITY OF THE COLORIMETRIC ASSAY

| MG IAA ADDED | MG PER 500 GM MEASURED | MG PER 500 GM MINUS BLANK | PERCENT ERROR |
|---|---|---|---|
| 0 | 4.0 | ... | .. |
| 12.7 | 16.5 | 12.5 | −2 |
| 19.7 | 24.0 | 20.0 | +2 |
| 12.8 | 16.2 | 12.2 | −5 |
| 18.6 | 22.5 | 18.5 | −1 |

DETERMINATION OF INDOLE-3-ACETIC ACID AND ITS ESTERS IN CORN KERNELS: Eight samples of corn kernels were collected at various stages of development from pre-fertilization through maturity. Immediately after harvest the kernels were cut away from the cobs, placed in sealed pliofilm bags, and stored at −25°C. For analysis, one kg (fresh weight) samples were macerated with ethyl acetate in a Waring blendor, and extracted as outlined above to estimate the quantity of IAA. Esters of IAA were determined first by removing the ethyl acetate in vacuo and then refluxing with 3% alcoholic KOH for 1.5 hours to hydrolyze the esters of IAA. The alkaline solution was neutralized to litmus with 10% HCl and the alcohol removed under reduced pressure. The residue was dissolved in ethyl acetate and the esters, as the equivalent of the IAA formed, were determined colorimetrically by the procedures previously described. The results of these determinations are tabulated in table II.

Dry weights were obtained by taking representative 20 gm fresh weight samples at each stage of maturity, drying at 70°C and 5 mm pressure, reweighing, and converting the fresh weights to the equivalent dry weights by these factors.

TABLE II

THE COMPARATIVE CONCENTRATIONS OF INDOLE-3-ACETIC ACID AND ITS ESTERS IN CORN KERNELS AT VARIOUS DEVELOPMENTAL STAGES

| STAGE OF DEVELOPMENT | μG IAA PER KG | μG IAA PER 100 MG | μG IAA ESTERS PER KG | μG IAA ESTERS PER 100 MG |
|---|---|---|---|---|
| | (*fresh weight*) | (*dry weight*) | (*fresh weight*) | (*dry weight*) |
| Pre-fertilization .. | 525 | 0.891 | None | None |
| Fertilization ..... | 525 | 0.891 | None | None |
| Blister ..... | 750 | 0.990 | None | None |
| Young milk ..... | 1275 | 0.768 | None | None |
| Intermediate milk | 2175 | 0.828 | 1500 | 0.571 |
| Late milk ..... | 1050 | 0.281 | 800 | 0.214 |
| Dough ..... | 870 | 0.219 | 560 | 0.141 |
| Mature ..... | None | None | None | None |

## SUMMARY

A colorimetric method for the separate determinations of IAA and its esters as they occur in developing corn kernels is presented. While some IAA was present at all stages except in the mature kernels, it reached a peak in fresh corn during the intermediate milk stage. The tomato fruit-setting factor, ethylindole-3-acetate was found only in kernels which had matured to—or beyond—the intermediate milk stage. The highest concentration of the ester occurred at the same stage of development which characterized the peak in concentration of IAA.

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## NEWS AND NOTES

The Chemical-Biological Coordination Center is reviewing the publication habits and related research practices of American biologists. In connection with this study the Center is seeking sources of unpublished data from research in all areas of biology where the effects of chemicals on biological systems are involved. The first returns from a nationally distributed one-page questionnaire have shown a most encouraging degree of interest. The Center is grateful for the cooperation it is getting, and urges that all biologists who receive a questionnaire during the next few months complete and return it as soon as possible.

The survey is intended to improve the efficiency of the CBCC as a research tool whose primary function is to coordinate and speed up the dissemination of facts and ideas from original research in the biological sciences. The results of the study will be analyzed and distributed to all those who are interested. Inquiries regarding the CBCC may be addressed to: Dr. Karl F. Heumann, Chemical-Biological Coordination Center, 2101 Constitution Avenue, N.W., Washington 25, D. C.

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*Western Section:* Officers of the American Society of Plant Physiologists, Western Section, for 1954:

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The Western Section of the A.S.P.P. plans to participate in the Pacific Division, A.A.A.S. meetings in Pullman, Wash., June 22–24, 1954. In addition to the presentation of papers during two days, two symposia are on the program. There are over 300 members of the Western Section, A.S.P.P.

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### RUFUS PERCIVAL HIBBARD

The members of the American Society of Plant Physiologists will learn with sorrow of the death of Dr. Rufus Percival Hibbard on August 26, 1953, in East Lansing, Michigan. Dr. Hibbard's name is intimately associated with the formative days of the society, being its first vice-president in 1924, and its second president in 1925. Through his untiring efforts and financial aid he helped in the organization of the society. His publications in the official journal were numerous and his generous contributions in an executive and editorial capacity were of great value to its success.

Dr. Hibbard was born in New Haven, Connecticut, on April 2, 1875, and came from a long line of Congregational ministers. The days of his youth were spent in Gloucester, Mass., where he acquired a love of the seashore which he retained throughout his life. His A.B. degree was obtained from Williams College in 1899, and he did graduate work at Harvard University, the University of Chicago, and the University of Michigan. He also studied at the Marine Biological Laboratory at Woods Hole. His first teaching assignment was at Kiskiminitas Springs Academy, Saltsburg, Pa., and later he taught at Blair Academy, Blairstown, N. J. He received his Ph.D. from the University of Michigan in 1906, and then became research assistant in the Bureau of Plant Industry, U. S. Department of Agriculture. From 1908 to 1911 he was bacteriologist and plant pathologist at the Mississippi Agricultural and Mechanical College, Starksville, Miss. He came to Michigan State College (then Michigan Agricultural College) in 1911, as assistant plant physiologist, and later became research associate and associate professor of plant physiology. He retired from this position in 1940, but during World War II he taught physics in the U. S. Army Air Force Training Program at Michigan State College.

His chief research interests were in the field of mineral nutrition and phytochemistry. He also published several articles on ripening, potato hollow heart, plant pigments and other subjects.

Dr. Hibbard was one of the "old school" plant physiologists, and his academic interests went beyond his professional field. His office door was never closed, and he always had a sympathetic ear for his students. Students usually left his conferences with encouragement and renewed vigor. The plant physiological seminars which he conducted in his home were looked forward to by students and colleagues alike.

Despite his very active life, he always found time for his family and his friends. In 1930, he took his family on a tour of Europe and attended the International Botanical Congress in Cambridge. He was very active in church work and in civic undertakings, and his cheerful advice was frequently sought after. Despite his quiet personality, he had a great driving force.

Plant physiologists have lost a great friend and scholar. His untiring service to the society and unselfish devotion to the field of plant physiology will long be felt. Dr. Hibbard leaves his widow, Dorothea Hibbard, and a son who is a research chemist with the Dow Chemical Company of Midland, Michigan.—HERBERT C. BEEKSKOW.

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## POSTSCRIPT

In January, 1926, volume 1, No. 1 of Plant Physiology appeared under the editorship of Charles A. Shull and an editorial board of Charles B. Lipman, Carleton R. Ball, Burton E. Livingston and Francis E. Lloyd. Dr. Shull was more than editor of Plant Physiology, for he was the moving spirit in the organization of the journal and Society, and he remained its editor through the year 1945. Fortunately for the Society, Dr. Shull planned his journal well, and it prospered under his direction and under the direction

of his successor, Dr. Walter F. Loehwing. The foreword which appears in volume 1, No. 1, sets out the program that the journal was to follow, and the new editor twenty-eight years later sees no reason to depart from the original program. However, our membership has grown from an initial one of 134 to over 1,000, and the number of papers appearing in the field of plant physiology is very much greater than it was twenty-eight years ago.

As is apparent, volume 29 of the journal comes out in a new format because the necessity of the situation requires it. The marked increase in the cost of printing, and the increasing number of papers being submitted, are such that it is not economically feasible to continue the format which has been so successful through the first twenty-eight volumes. The new format is such that we can publish about twice as much material per page as was possible in the earlier one, with some sacrifice in appearance and in margins. One page of text is not quite equal to two pages of the old format, and considerable savings will result in the bill for paper and mailing. Actually, the new format will probably produce larger savings in the handling of tabular material and figures than in any other field.

The editor is largely responsible for the choice of the particular format, since the pressure of his duties, on top of a normal University life, have been such that he did not have time to circulate the new format to all the members of the editorial board and the officers of the Society. He hopes, however, that even though individuals may not agree with particular details of the present appearance of Plant Physiology, members will recognize that the new format is one which was forced upon us for economic reasons.

The basic editorial policy of Plant Physiology is summarized in two paragraphs taken from the foreword published in No. 1 of volume 1, which are given below:

> This journal, therefore, exists for the general advancement of this great field of investigation, for all of the plant sciences where a physiological attack must be employed in the solution of the problems encountered. It hopes that it may serve as an instrument for the integration and unification of all plant physiologists into a powerful working unit, without interfering with the organized activity of other groups, by providing a central medium through which we can cooperate in the advancement of plant physiology, and of the welfare of plant physiologists, in all fields of endeavor. To this end it invites the support of plant physiologists of every denomination, "fundamentalists and modernists", pure physiologists and applied physiologists. It has no other purpose, and no other desires than to be of service, and to promote cooperation in the common tasks of advancing plant physiology as a pure and applied botanical science. We extend this invitation with confidence that many hands will lend themselves to lighten the burdens which have been laid upon the editorial committee".

One must recognize that Plant Physiology has changed in the last twenty-eight years in detail but not in basic viewpoint. More papers are appearing in which biochemical methods are used to obtain the data, and more papers are appearing in which mathematical analysis is essential for the interpretation of physiological results than was formerly true. The publication of mathematical and chemical formulae has itself increased publication costs.

The increase in the development of our science will inevitably mean more rather than less of mathematical analysis and the use of formulae in manuscripts.

As was pointed out by the editor twenty-eight years ago, we are interested in articles in Plant Physiology that contribute to physiological science without reference to the department of origin or to the professional title of the author. Good physiology is often done on plants of economic importance, and there is at least as much reason for publishing papers dealing with physiology of corn or sugar beets as for some other organism. However, the number of manuscripts received by Plant Physiology is so great that papers which are primarily agricultural, that is, that deal with the increased yield of a particular crop, should be submitted to agricultural journals rather than to Plant Physiology.

The nature of the development of the science of plant physiology is such that we must expect in the future that articles will occasionally appear which are theoretical, that is, the reinterpretation of previously published material and do not contain new data of their own. The journal should be responsive to such articles, provided that the same standards of accuracy and quantitative thinking are insisted on in a theoretical article as an experimental one. The authors of such articles should expect theoretical papers to be sent out to reviewers and that the theory should be checked against the existing body of scientific knowledge. We cannot afford to give space to mere speculation that is not based upon an established body of scientific knowledge.

As the mass of information in plant physiology accumulates, we must recognize that mere measurement alone does not constitute scientific investigation. Biology is so broad that the number of possible measurements that can be made is extremely great, and the measurements may turn out to be of use to biology only if they are of value in the extension of scientific knowledge, in the confirmation of hypothesis or in the demonstration of the validity of a particular theory. Further, physiological data is frequently printed which would be far more useful if a time axis were also presented, so that instead of merely having a static picture of the composition in one cross-section of time it were possible to calculate the changes in composition which occur through time. In the same vein, the presentation of data on the composition of developing fruits and seeds merely as the percentage of protein, for example, without any measure of the number of fruits and seeds analyzed, makes it impossible for the interested reader to recalculate the amount of protein per fruit or seed as it develops. Some work and thought on these problems on the part of authors would increase enormously the use to which their own

scientific data could be put in the development of their own science.

One member of the editorial board remarks that a paper "should tell a story," and there is much merit in this proposal so that scientific data are presented in a consistent picture and an article develops a thesis. This suggestion will not be valid if the story is forced or merely glib.

Lastly, the new editor wishes to thank the members of the Executive Committee of the Society for entrusting the journal to his hands, and to the members of the editorial board, the numerous reviewers and the authors for the help they have given him in the past few months. The delay in the appearance of the journal is unfortunate, but has been forced by the pressure of circumstances at this time. It will probably be early summer before our journal will appear regularly as a bi-monthly, and the editor asks for the forbearance of the members in this regard for the immediate future.

DAVID R. GODDARD

## PREPARATION OF MANUSCRIPTS FOR PUBLICATION IN PLANT PHYSIOLOGY

The following suggestions are made to authors writing papers for this journal in order to minimize revisions and editorial corrections. Though many points may appear arbitrary or trivial, attention to details is necessary to secure uniformity of format and style. The membership dues and subscription fees go almost entirely to publication costs, as all of our editors give their services without financial recompense. Hence the cooperation of authors in preparation of manuscripts minimizes expense and permits greater liberality as to length and number of papers published. Authors will find the following concise publications useful in preparation of manuscript copy: A. J. Riker, The Preparation of Manuscripts for Phytopathology. PHYTOPATHOLOGY XXXVI, pages 953–977, 1946; and S. F. Trelease, The Scientific Paper: How to Prepare It. Second Edition. Williams and Wilkins Company, Baltimore, Maryland, 1951.

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